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Grading in superficial bladder cancer: morphological and biological parameters

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God zij gedankt dat ik in die tijd van mijn meester
de wil om te leren
en het gevoel voor de rechte weg verwierf,
welke men behoudt,
ook wanneer het pad kronkelig is.

Umberto Eco: De Naam van de Roos.

Contents

Glossary and abbreviations

TCC : Transitional Cell Carcinoma
TURT : Transurethral Resection of Tumor
FCM : Flowcytometry
BGI : Blood Group Isoantigens
SRCA-test : Specific Red Cell Adherence-test

TNM Staging System [1]

T : Primary Tumor
N : Regional and Juxta-regional Lymph Nodes
M : Distant Metastases
Tis : Carcinoma in situ, pre-invasive
Ta : Papillary non-invasive carcinoma
T1 : The tumor does not invade beyond the submucosal connective tissue
T2 : There is microscopic invasion of superficial muscle
T3 : Invasion of deep muscle or extension through the bladder wall
T4 : Tumor fixed or extending to neighbouring structures

The suffix (m) may be added to the appropriate T category to indicate multiple tumors, e.g. T2 (m).

WHO Grading System [2]

G1 :Low grade tumors with the least degree of cellular anaplasia, compatible with a diagnosis of malignancy
G2 :Intermediate degree of cellular anaplasia
G3 :High grade tumors with the most severe degree of cellular anaplasia

Some examples of cytogenetic nomenclature [3]

Karyotype :	A systematic arrangement of the chromosomes into various groups according to size, centromere location and banding patterns obtained after differential staining procedures
47,XY,+21 :	47 chromosomes, XY sex chromosomes and an additional chromosome # 21
45,XX,-8 :	45 chromosomes, XX sex chromosomes and a missing chromosome # 8
47,XY,+14p+ :	Male karyotype with 47 chromosomes including an additional chromosome # 14 which has an increase in the length of its short arm
47,XX,+1q- :	Female karyotype with 47 chromosomes including an additional chromosome # 1 which has a loss of length of its long arm
Chromosome#1 :	Chromosome number 1
2n :	Diploid number of chromosomes, in the human beings 46
del :	Deletion, loss of a chromosome part
t(8;14) :	Translocation between chromosomes#8 and #14; breakage, followed by exchange of chromosome material between these chromosomes

Triploid .	All the chromosomes are present in the triplicate, i.e 69 chromosomes (3n)
Near diploid :	The modal number cannot be given as a precise number (2n ±)
Pseudodiploid:	The modal number of chromosomes is equal to the diploid number (for human beings 46), but numerical and/or structural aberrations are present

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3 **ISCN (1978)** An International System for Human Cytogenetic Nomenclature (1978) Birth Defects Original Article Series, Vol 14, No 8 (The National Foundation, New York 1978), also in Cytogenet Cell Genet 21 309-404 (1978)

Chapter I

Introduction

Histopathological prognostic factors in bladder cancer.

Bladder cancer, after prostatic cancer the most common cancer seen in urology[1], can basically be segregated in three forms: the papillary transitional cell carcinoma, a deeply invasive form that often disseminates, and carcinoma in situ [2-6]. The most important single factor affecting prognosis is the extent of spread or stage of the tumor. [3,4,7-13]. The depth of invasion in the bladder wall or into adjacent tissues by the neoplasm correlates with the presence of metastases, and this implies the potential for cure. [8,10,14-16] However, in the majority of patients presenting for the first time with bladder cancer, the tumor is papillary, superficial and shows no invasion or only invasion in the submucosal connective tissue. [4,17] Most (50 - 80%) of these tumors recur and some, varying from 3 per cent for Ta tumors to 44 per cent for T1 tumors, do so in a more advanced, potentially lethal stage. [2,17-21] Therapeutic efforts are directed towards conserving the bladder as long as possible, recognizing that although bladder tumors recur frequently, many patients will have an indolent clinical course. Common practice is to delay radical therapy (radiotherapy and/or cystectomy) until muscle invasion has been clearly documented. Unfortunately, when this event has occurred, five-year survival is only 40 to 50 per cent regardless of the use of preoperative radiation or the extent of surgical extirpation. [6,10,13,22-24] Systemic dissemination has already occurred in many of these patients and is reflected in the appearance of distant metastases within 18 months. [25] So the heterogeneity of superficial bladder tumors is clinically manifest in varying patterns of recurrence and prognosis, and in varying responses to surgical extirpation, radiation or chemotherapy. Therefore, in transitional cell carcinoma, the pathologist, in addition to his task of establishing the diagnosis, is asked to predict the biologic potential of the tumor. He than has to identify patients with non-invasive disease with the greatest risk of recurrence with or without invasion, and to identify patients with invasive disease with the greatest risk of dissemination and death of tumor. To allow the pathologist to make an accurate statement with respect to the depth of invasion of the tumor, a biopsy must include a sufficient amount of muscle tissue. A superficial transitional cell carcinoma (T.C.C.), the object of this thesis, has an exophytic, papillary growth pattern. Even in a deep biopsy specimen including muscle, the recognition of invasion of the lamina propria may be difficult for the pathologist. [26] So there is a need for other prognostic criteria, additional to tumor stage.

Additional histopathological characteristics reported to have predictive value are tumor grade [27,28], number and size of visible tumors [2,21] associated mucosal abnormalities elsewhere in the bladder [17,20,29] and for tumors once recurring a high recurrence rate [30] and the presentation of higher graded recurrences. [31,32] Of all these parameters, tumor grade is the most important.

The concept of tumor grade was introduced by Broders [33,34] who subdivided squamous cell carcinoma in four groups, depending on the degree of differentiation of the cells. Grading of bladder tumors appeared to be of great prognostic value. [27,28,32] For bladder cancer, several grading systems have been described. [26-28, 34-37] Generally transitional cell carcinoma of the bladder is divided in three grades; grade 1 or low grade carcinoma, grade

2 carcinoma of intermediate grade, and grade 3 or high grade cancer. When a fourth grade is used this applies to undifferentiated tumors. Either these grade 4 tumors are not recognized as primary urothelial tumors, or they should be classified as TCC grade 3. [28] As not everyone accepts the same pathological criteria for the diagnosis of malignancy [26], the definition of what is a grade 1 or low grade tumor varies. For some a grade 1 papillary lesion shows only thickened urothelium, without significant nuclear abnormalities. [27,28] For others grade 1 applies to tumors that have "the least degree of cellular anaplasia compatible with a diagnosis of malignancy" [26] or nuclear pleomorphism and disturbance of cellular architecture. [37] Generally, the clinical course of grade 1 tumors is rather innocent, for grade 3 tumors poor. There appears to be a variable interpretation of the intermediate grade 2 neoplasms. Often pathologists classify tumors as being grade 1-2, or grade 2-3, in correlation with the clinical presentation of "good" and "bad" tumors of moderate grade. [28] Even when using the same grading system, the grade given to a particular tumor may vary between different pathologists, and occasionally for the same pathologist on different days. [38] The study by Ooms and associates [38] revealed that about 50 per cent of all tumors were graded in a significant different manner by the same reviewer after 7 months. Thus grading is rather subjective, requiring a referee pathologist in comparing final results of different treatment modalities, or of the same treatment in different institutions. These problems of terminology and interpretation for many years hampered communications about superficial bladder tumors between urologists and pathologists of different institutions. Much more information about the biology of bladder cancer is needed to support the morphological criteria like low and intermediate grade. The variability in tumor grading, and especially the heterogeneity of superficial intermediate grade tumors prompted this study for a reliable and reproducible histopathological, clinically relevant distinction within these grade 2 neoplasms. That is one objective of this thesis.

Additional tumor markers

The shortcomings of traditional histopathological parameters in the heterogeneous group of superficial bladder cancers made additional tumor markers urgently needed. Tumor markers can be substances produced by the tumor itself and secreted into the body fluids. As such markers in TCC were mentioned for instance carcinoembryonic antigen [39] polyamines [40], rheumatoid factors [41], fibrin degradation products [42] and tryptophan metabolites. [43] Most of the putative tumor markers detectable in blood or urine have not fulfilled their earlier promise in bladder cancer. [42,44-51] Tumor markers also may be noncirculating substances, confined to the malignant cell itself. As most sensitive cellular tumor markers in bladder cancer were reported chromosomal abnormalities [48,52-54], cytometric DNA-values [55-60], and the ABO (H) blood group antigens. [61-65]

Chromosomes and bladder cancer

The idea that chromosomes play a role in malignant cell growth is an old one. [66-69] Cytogenetic experts were only able to recognize chromosomal abnormalities in tumors in a reliable and reproducible way after the introduction of differential staining techniques. Microscopic analysis of the banded chromosomes is most easily done in the metaphase. Terminology and classification of chromosomes and their abnormalities were standardized and summarized in "An International System for Human Cytogenetic Nomenclature". [70] The chromosomes are numbered and divided into seven groups (A -

G), each group with chromosomes that are morphologically more or less similar. Banding techniques make identification of individual chromosomes possible, and can be used to demonstrate abnormalities of chromosomes that seem normal with conventional staining methods. [71,72] Several techniques frequently used are:
G-banding: named after Giemsa, the most widely applied stain; this method produces light and dark bands which are visible with the light microscope, after various forms of pretreatment;
Q-banding: more or less the same type of pattern can be produced, without pretreatment, by Quinacrine (mustard) stain, only visible however in fluorescence microscopy;
C-banding: method to stain the constitutive heterochromatine which is located around the centromeres and in the distal part of the long arm of the Y chromosome and can vary among individuals.
The cytogenetically recognisable abnormalities in tumors are of three kinds:
- numerical abnormalities i.e. any abnormality in the number of chromosomes, (aneuploidy; polyploidy);
- structural abnormalities; a chromosome resulting from a structural abnormality that cannot be identified, is called a marker chromosome;
- fragmentation of chromosomes in minutes and double minutes.

Well known examples of chromosomal abnormalities that are more or less specific for a certain neoplasm are the Philadelphia chromosome, which is the result of a translocation between chromosome number 22 and number 9 in chronic myeloid leukemia [73], and the translocation t(8;14) in the Burkitt lymphoma. [74]

The first report about cytogenetic analysis in bladder cancer cells was made by Spriggs and associates [75], mentioning chromosomal abnormalities in peritoneal effusions of two patients. Larger series followed soon. [76,77] An increase in chromosome number appeared associated with higher graded tumors, malignancy and infiltration. [77,78] Structural abnormal chromosomes were found [77] which, in superficial bladder cancer, were mentioned as reliable prognostic parameters in individual patients. [52-54,79] Cytogenetic examination of bladder carcinoma may therefore in a very early stage produce a most sensitive and specific tumor marker with respect to future behavior. The reported correlation between cytogenetic data and tumor grade, and the biologic potential for recurrence and invasion led to the decision to study chromosomal abnormalities as an objective basis for grading or prognosis.

Karyotypic changes can be a reflection of alterations in the location of oncogenes. [73,74,80-86] These karyotypic changes can occur as translocations, amplifications, deletions or fine structural alterations. They are at least markers of essential genetic events. Primary karyotypic changes are possibly an expression of primary oncogenetic processes, of importance in the etiology of the cancer [87] Specific karyotypic aberrations can also be secondary and reflecting tumor events or chromosomal selections later in time, parallel to the biological behavior of tumors as their capacity to invasion, metastatic spread and reactions to therapy. [88] Recently in bladder tumors nonrandom chromosomal changes have been demonstrated. [89] Until now cytogenetic analysis in bladder cancer did not identify the earliest irreversible changes predicting invasion and metastasis. Therefore banding techniques were applied in a number of bladder tumors described in this thesis to see whether nonrandom abnormalities in the chromosomes could be demonstrated.

Preceding the analysis of chromosomal changes in bladder cancer, a useful technique to obtain recognizable chromosomes from as many tumor specimens as possible was developed. This technical aspect is reported elsewhere. [90,91] In this thesis the correlations between cytogenetic data, tumor grade, and the biological behavior of superficial bladder cancer are analyzed.

ABH antigen deletion

Another biological marker which is reported to reflect cellular differentiation and the malignant potential of a tumor is the presence or absence of certain cell surface antigens such as the ABH blood group isoantigens (BGI). In normal urothelium the cell membranes are rich in ABH blood group antigens [92,93] and it was suggested that the presence or absence of these substances in TCC might have prognostic value. [61-63,94,95] Loss of these isoantigens in metastatic bladder carcinoma appeared to be quite common. [62] This however is not the case with nonmetastatic disease. In superficial TCC of the bladder loss of these antigens is stated to occur especially in tumors which carry a poor prognosis with respect to invasion and metastasis. [63,65,96-98] Catalona [64] in a survey on studies on BGI-deletion, concluded that testing for blood group antigens was not useful in predicting recurrences. Most authors mentioned however a clear correlation between antigen content and other aspects of tumor behavior. Thus, at the start of our study on ABH antigen status in bladder tumors, it was generally believed that the determination of ABH antigen expression in urinary bladder carcinoma might be helpful in discriminating between those patients with superficial bladder carcinoma who are at low risk for subsequent tumor invasion from those at relatively high risk, for whom early aggressive therapy might be justified.

However, studies on ABH antigen status had serious limitations. In many of these studies the proportion of low grade, low stage carcinomas that later became invasive was surprisingly high, compared with the percentage seen in common clinical practice. [4,17] In addition, the lack of satisfactory methodology for detecting the antigens in tissue sections, and the subjectivity in the interpretation of test-results hampered wide-spread clinical implementation of assessing BGI deletion in bladder tumors.

The initial studies on BGI deletion employed the specific red cell adherence (SRCA) test, developed from the mixed red cell agglutination test. [99] This test involves adherence of erythrocytes to the appropriate antigens in a tissue section. Since antibody to antigen H in the membranes of blood group O patients was not readily available, a lectin was substituted, extracted from the seeds of Ulex Europaeus, a leguminous shrub. [100,101] This lectin crossreacts with the weakly immunogenic antigen H and therefore can replace the antibody for antigen H. The relatively small size of the Ulex lectin molecule that is used to detect antigen H makes the SRCA-test less sensitive in blood group O patients with much more false negatives, than is the case for patients with blood group A and B. [98,102] In addition the SRCA-technique gives a limited resolution because of the large size of the indicator particles, erythrocytes. Therefore the test is difficult to standardize and interpret.

Because of the limitations of the SRCA-technique, we preferred the immunoperoxidase method. [103,104] This technique offers an increased sensitivity for the H antigen and a superior resolution. A practical advantage of the immunoperoxidase procedure is that this method lends itself to the processing of large batches of slides simultaneously. It was already routinely used in our pathology laboratory to demonstrate hormones and other

substances in tissue sections. However, also in the immunoperoxidase method Ulex lectin was routinely used for detection of the H tissue antigen. With this method anti-lectin-antiserum is the secondary antibody, linking with the horse-radish peroxidase chromophore third antibody. This lectin-antiserum presumably combines at multiple sites on the lectin molecule which makes the sensitivity of the immunoperoxidase technique greater. [105] To our great disappointment the need to use a lectin in detecting the H isoantigen, together with variation in specificity found with conventional polyclonal blood group antisera, limited the reliability and reproducibility of the immunoperoxidase technique. Conventional polyclonal antisera such as those obtained from the immunisation of animals invariably consist of a heterogeneous mixture of antibodies to more than one antigen.

In 1975 a technique was described to produce monoclonal antibodies (McAb's) with the use of immunoglobulin-secreting mouse hybridoma cell lines. [106] This led to the production of pure, homogeneous and highly specific reagents to the A, B and H isoantigens. Experience with the use of McAb's as primary antibodies in larger series of bladder cancer was not yet available. So we started a study on BGI-deletion in superficial bladder cancer using the indirect immunoperoxidase method with McAb's, to see whether testing for ABH-antigen status could fulfil the earlier promise of producing an additional tumor marker in superficial TCC. Our original goal was to examine, at first tumor presentation, an additional parameter to predict future tumor behavior. In this thesis presence or absence of blood group isoantigens in superficial TCC of the bladder is correlated with tumor stage and grade, chromosomal numbers and clinical course.

Aims of the present study

Primary aim of this study was the investigation of predictors of the clinical course additional to tumor stage, in superficial transitional cell carcinoma of the bladder. Because of the variability of grading a prospective study was carried out in which the intermediate grade tumors were separated in two subgroups to see whether it is possible to make a sharper histopathological, clinically relevant distinction between low and intermediate grade bladder tumors.
A study of the chromosomes in bladder cancer was done in order to obtain more information about biological characteristics of these tumors, to investigate the prognostic value of chromosomal abnormalities, and to examine whether a correlation between our grading criteria and the results of cytogenetic analysis can be demonstrated.
Next, an analysis of cellular ABH blood group antigens in bladder cancer was done, using the indirect immunoperoxidase technique with monoclonal antibodies. This was correlated with tumor grade, chromosomal number and clinical course in superficial tumors. Aim was to investigate whether with the use of this essay a correlation was demonstrable between ABH antigenstatus and our grading criteria, or whether ABH antigen- testing had additional prognostic value.

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Chapter II

Cytogenetic analysis in urothelial cell carcinoma

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Abstract

Tissue specimens from 96 patients with urothelial cell carcinoma were subjected to cytogenetic examination to determine whether there is a correlation between chromosomal abnormalities and tumor behavior. Recognizable metaphases were found in 43 patients (45 per cent). The range in chromosomal counts appeared to be a better reflection of invasion than the modal number. Noninvasive tumors nearly always were hypodiploid or diploid. All invasive tumors showed hyperploid cells. Cytogenetically, there was no difference between invasive grade 2 and grade 3 tumors. During our limited follow- up tumor progression was found only in tumors with hyperploid cells. With a direct technique we found no difference in the frequency of marker chromosomes according to tumor stage and grade.

Introduction

Transitional cell carcinoma of the urothelium has an unpredictable natural history with respect to recurrence and invasiveness of superficial lesions.[1] In daily urological practice the most important predictors of the clinical behavior of urothelial cell carcinomas are tumor stage and grade. However, the histopathological terminology is not well standardized, and cannot predict which superficial bladder tumors will recur and become invasive.[2] An additional parameter for the prognosis might be obtained from cytogenetic studies.[3,4] The probability of recurrence or invasion may be linked to the presence of numerical or structural chromosome abnormalities. In early studies an increase in modal chromosome number (hyperploidy) was associated with malignancy and infiltration.[5,6] Spooner and Cooper found that well and moderately well differentiated tumors fell within the diploid range (42 to 49 chromosomes), whereas the majority of poorly differentiated cancers showed a widespread distribution of chromosome numbers.[6] Falor[7] and others[4,8,9] found a large number of structural abnormalities, including marker chromosomes, especially in poorly differentiated and invasive tumors. Low stage, low grade carcinomas of the bladder nearly always were diploid or near diploid (42 to 49 chromosomes), although a few marker chromosomes were observed.[4,10]

In regard to the prognosis in superficial bladder carcinoma, Summers and associates concluded that patients with hyperploidy especially appear to be at a high risk for subsequent invasion.[4] Granberg-Öhman and associates stated that tumors with a diploid mode also could become progressive.[9] Many investigators have stressed the prognostic importance of marker chromosomes, especially in tumors with a chromosome number in the diploid range.[3,8,9,11] Falor and Ward mentioned that the modal number of the tumor so closely mirrors the presence of markers that the number hardly affords additional information with respect to the prognosis.[11] This discrepancy as to the meaning of chromosomal abnormalities, especially in low stage, low grade bladder tumors, clearly shows the need for more follow up to reveal the biological significance of the reported chromosomal aberrations.

We performed chromosome analysis in relation to stage, grade and tumor behavior.

Materials and methods

Between January 1981 and June 1984, tissue specimens from 96 patients with transitional cell carcinoma (91 in the bladder, 3 ureter and 2 pelvis) were subjected to chromosomal

examination. The patients were followed until December 1985, or until tumor progression or treatment with intravesical chemotherapy. Included were 14 patients who already had a recurrent tumor. None of these patients had been treated previously with radiotherapy or intravesical chemotherapy. Tissue was taken by transurethral resection, as well as by cold punch biopsy. A portion of each biopsy was sent to the pathology anatomy laboratory and another was sent to the cytogenetic laboratory.

A direct technique was used for chromosomal analysis and, if enough tumor material was available, a short-term culture was applied. The detailed procedure with an evaluation of the techniques used has been reported previously.[12] For the direct method material was collected in 0.9 per cent sodium chloride with 1 μg./ml. colchicine. After incubation for 30 minutes at 37°C, mechanical disaggregation of the tissue and preparation of a cell suspension were done. Hypotonic treatment in 0.075 M. potassium chloride was followed by fixation in methanol-acetic acid 3:1. During the last year a direct squash technique was used.

As a short-term culture the material was incubated for 24 hours in tissue culture RPMI-1640 medium plus 15 per cent fetal calf serum and 4 per cent penicillin-streptomycin (5,000 units per ml.) at 37°C. Two hours before termination of the culture 0.1 μg./ml. colchicine was added, followed by hypotonic treatment and fixation. The preparations were stained with the Giemsa method. If sufficient slides were available and the unbanded preparations showed good metaphases, C.B.G.-banding and G.T.G.-banding methods were used. Normal bladder tissue was rather solid and usually could not be worked well into a cell suspension for the direct technique. The specimen was subjected to a long-term culture in Ham's-F10 medium with 15 to 25 per cent fetal calf serum in a 5 per cent carbon dioxide and 95 per cent air atmosphere for 1 to 4 weeks. Analyzable metaphases were photographed and karyotyped according to the Denver and Paris nomenclature.[13] Cells in metaphase were considered to be analyzable if there were none or only a few overlapping chromosomes and the number of chromosomes at least could be counted. If the number of chromosomes was in the diploid range it was considered necessary to recognize the A to G groups.
According to the chromosome number the tumors were classified as to 1) the modal numbers, that is hypodiploid (less than 46 chromosomes), diploid (46), hyperdiploid (47 to 57), near triploid (58 to 80), near tetraploid (81 to 103) and hypertetraploid (more than 103), and 2) whether the tumors had cells only in the hypodiploid or peridiploid range (49 or less), and cells with more than 49 chromosomes (hyperploid). Marker chromosomes were classified according to the position of the centromere.[14]

Clinical staging of the tumor was done according to the tumor, nodes, and metastasis classification,[15] and grading was done according to the World Health Organization grading system.[16]

Results

A summary of the 96 cases according to tumor stage and grade, and the number of patients with countable metaphases is shown in table 1. When applied, the direct and the short-term culture techniques revealed identical chromosomal abnormalities. Therefore, these results were pooled. The number of metaphases varied between 3 and a large amount per tumor, or between 1 and approximately 25 per slide.

Table 1
Patients examined and countable metaphases

	Stage					
	pTa	pT1	pT2	pT3	pT4	Totals
Grade 1	4/-	-/-	-/	-/-	-/-	4/-
Grade 2	48/18	14/5	3/2	9/7	-/-	74/32(43)
Grade 3	1/	4/3	4/4	8/4	1/-	18/11(61)
Totals	**53/18(34)**	**18/8(44)**	**7/6(85)**	**17/11(65)**	**1/-**	**96/43(45)**

Values are given as number of patients examined/number of patients with recognizable chromosomes (per cent of those examined)

Countable metaphases were found in 43 of 96 tumors (45 per cent). During the last year of our study this value increased to 70 per cent. However, mitoses that included metaphases not suitable for further chromosomal analysis were seen in 70 of the tumors examined (73 per cent)· 34 of 53 (64 per cent) noninvasive and 36 of 43 (84 per cent) invasive tumors. Multiple tumors did not show mitoses more frequently than the single carcinomas even if more biopsies were done Usable metaphases were seen in 18 of 53 (34 per cent) non-infiltrating, 8 of 18 (44 per cent) superficially invasive and 17 of 25 (68 per cent) deeply infiltrating tumors. These results show a tendency that in deeper growing tumors (stages T2 and T3) the yield of recognizable metaphases is higher (chi-square test for trend, $p = 0.001$). A same tendency was seen for higher grade tumors. Among the 18 non-invasive tumors (stage pTa) mostly hypodiploid of diploid metaphases were noted (tables 2 and 3). The 8 superficially infiltrating tumors (stage pT1) showed a wide range in modal number. The 17 deeply infiltrating tumors (stages pT2 and pT3) were mostly in the triploid or tetraploid range, although 4 showed a diploid modal number Of the 32 moderately well differentiated tumors 21 were hypodiploid or diploid Among the grade 2 tumors the noninvasive lesions were mostly but not all hypodiploid or diploid, while the invasive tumors showed a wider range in modal number
Separately, a distinction was made in tumors with cells only containing 49 or less chromosomes, and in tumors with at least 1 cell containing more than 49 chromosomes. Of 18 non-invasive tumors 14 had only cells with 49 or less chromosomes (table 3).

Table 2
Cytogenetic data in 43 patients

Pt No	Stage	Grade	No Countable Metaphases (range)	Modal No	Ploidy (n = 23 ± 1 etc) n	2n	3n	4n	5n	Marker*	Banding-Technique**
1	Ta(m)	2	3(46)	46	-	3	-			-	
2	Ta	2	4(46)	46	-	4		-	-	-	-
3	Ta(m)	2	3(36-45)	45	-	3		-	-	submedian	-
4	Ta	2	4(30-46)	43	1	3		-	-	-	-
5	Ta	2	4(35 48)	46		4	-			submedian	-
6	Ta	2	7(43-46)	44	-	7	-	-	-	-	C/G
7	Ta	2	5(45 46)	46	-	5	-	-	-	-	
8	Ta	2	75(32-49)	46	1	74	-	-	-	submedian and median	C
9	Ta	2	10(34 46)	46	-	10		-		-	

Pt. No.	Stage	Grade	No Countable Metaphases	Modal		Ploidy (n = 23 ± 11etc)					Banding-
			(range)	No.	n	2n	3n	4n	5n	Marker*	Technique**
10	Ta(m)	2	46(38-46)	46	-	46	-	-	-	-	C/G
11	Ta	2	13(36-46)	44	-	13	-	-	-	-	-
12	Ta	2	5(42-44)	43	-	5	-	-	-	-	-
13	Ta	2	8(46-48)	48	-	8	-	-	-	-	-
14	Ta	2	35(23-44)	40	4	31	-	-	-	submedian	G
15	Ta	2	25(40-90)	43	-	19	1	5	-	submedian	-
16	Ta(m)	2	16(35-71)	43/70	-	8	8	-	-	-	-
17	Ta(m)	2	22(38-78)	43	-	19	3	-	-	-	-
18	Ta	2	13(50-90)	67	-	2	7	4	-	subterminal	-
19	T1(m)	2	11(35-72)	65	-	4	7	-	-	submedian, median and terminal	-
20	T1	2	16(28-73)	53	2	9	5	-	-	subterminal	-
21	T1	2	40(35-72)	42	-	38	2	-	-	submedian	-
22	T1	2	50(34-56)	44	2	48	-	-	-	subterminal and median	C
23	T1(m)	2	48(46-92)	46	-	38	6	4	-	-	-
24	T1	3	6(70-89)	80	-	-	3	3	-	terminal	-
25	T1	3	3(90-95)	92	-	-	-	3	-	-	-
26	T1(m)	3	15(23-80)	46	4	7	4	-	-	-	-
27	T2	2	33(43-75)	46	-	27	6	-	-	-	-
28	T2	2	18(38-78)	70	-	6	12	-	-	submedian, subterminal and terminal	-
29	T2	3	6(50-93)	69	-	3	-	3	-	terminal	C
30	T2	3	25(50-220)	115	-	-	-	15	10	subterminal and terminal	-
31	T2	3	29(36-90)	69	-	17	2	10	-	median	
32	T2	3	6(42-70)	46	-	5	1	-	-	-	-
33	T3(m)	2	4(41-56)	46	-	4	-	-	-	terminal	-
34	T3	2	3(46-100)	69	-	1	1	1	-	-	-
35	T3	2	13(65-80)	73	-	-	13	-	-	subterminal	-
36	T3	2	3(90-95)	92	-	-	-	3	-	-	-
37	T3	2	10(40-62)	46	-	9	1	-	-	-	-
38	T3	2	14(42-90)	69	-	10	-	4	-	-	-
39	T3	2	8(46-90)	69	-	6	-	2	-	-	-
40	T3	3	3(50-90)	69	-	1	1	1	-	terminal	-
41	T3	3	5(42-90)	69	-	3	-	2	-	-	-
42	T3	3	10(64-90)	75	-	-	5	5	-	-	-
43	T3	3	21(37-90)	90	-	4	4	13	-	-	-

* Unidentifiable structurally abnormal chromosome with the centromere in the submedian, median, subterminal or terminal region

** Procedures of differential staining technique by which various intrachromosomal bands and/or regions can be made visible, such as G-banding by Giemsa staining
C-bands are dark staining condensed regions corresponding in position mainly to the centromeric region.

(m): Multifocal

Table 3

Tumor stage and grade in relation to modal number of chromosomes and to number of chromosomes in individual cells

	Total No.	Stage			Grade 2		Grade 3
		pTa	pT1	pT2-pT3	Non-invasive	Invasive	
Total No. tumors	43	18	8	17	18	14	11
Modal No..							
Hypodiploid	10	8	2	-	8	2	-
Diploid	13	7	2	4	7	4	2
Hyperdiploid	3	2	1	-	2	1	-
Near triploid	13	1	2	10	1	6	6
Near tetraploid	3	-	1	2	-	1	2
Hypertetraploid	1	-	-	1	-	-	1
No. chromosomes:							
≤ 49	14	14	-	-	14	-	-
> 49	29	4	8	17	4	14	11

All 25 superficially (stage pT1) or deeply (stages pT2 and pT3) infiltrating tumors had hyperploid cells. The difference in chromosome counts according to this distinction, between non-invasive (stage pTa) and invasive tumors, is highly significant (Krushal-Wallis test $p = 0.0002$). According to the number of chromosomes no significant difference between submucosal (stage pT1) or deeply infiltrating tumors was found. All invasive grade 2 carcinomas showed cells with more than 49 chromosomes, as did all grade 3 tumors. Of the 18 stage pTa grade 2 tumors, 4 were hyperploid (more than 49 chromosomes). Patient 18 had inoperable disease and did not tolerate intravesical chemotherapy. Patient 15 had a follow up of 13 months and no recurrence. The other 2 patients had recurrences (patient 17 with progression to a stage pT1 tumor after 50 months of follow up).

Marker chromosomes

Marker chromosomes were established by conventional chromosome staining without special banding techniques and were found in 18 of the 43 patients: 6 of 18 (33 per cent) with stage Ta and 12 of 25 (48 per cent) with invasive tumors (table 2). There was no difference in the frequency of markers according to tumor grade.

In 8 patients a large marker was found with the centromere in the submedian region. This chromosome varied in size compared to the A1 chromosome (fig.1). A loss of an A and C-group chromosome often was associated with the presence of this marker. In patient 14 the marker was banded. Unfortunately, the pattern could not be interpreted fully. The markers with the centromere in the subterminal and terminal regions were found mostly in more infiltrating tumors (fig.2). The markers with the centromere in the median region were found in noninfiltrating as well as infiltrating tumors.

Min, and dmin and ring chromosomes

Patient 28 had some dmin, and patient 31 had many min and dmin chromosomes in the specimens. A small ring chromosome was present in the tissue of patient 30.

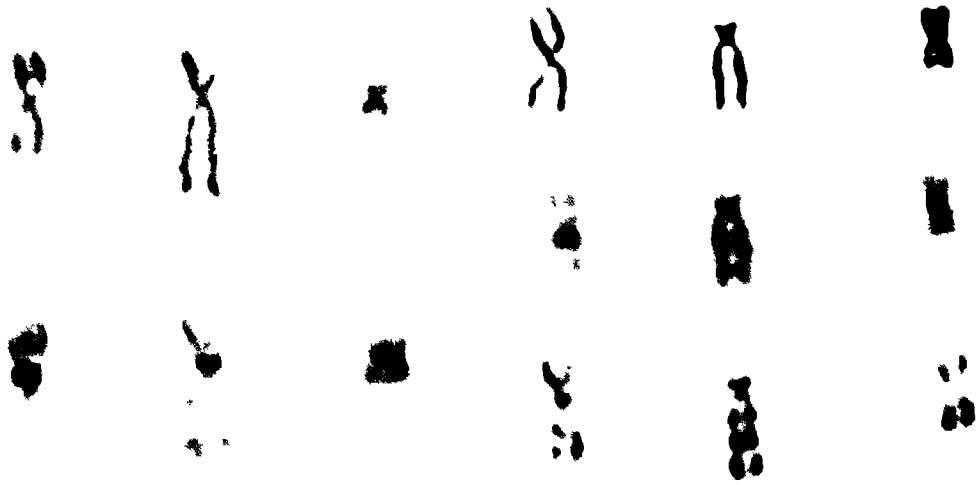

Fig. 1
Details of unbanded and C-banded karyotype of patient 8.
To the left for comparison of the size chromosome A1. To the right are two markers: sm and m.

Fig. 2
Details of unbanded, C-banded and G-banded karyotype of patient 22.
To the left for estimation of the size chromosome A1. To the right are two markers: st and m.

Normal bladder tissue

Random biopsies from normal bladder tissue in 11 patients with non-infiltrating tumors showed neither histopathological nor cytogenetic abnormalities.

Tumor recurrence and progression

Of the 18 patients with a stage pTa tumor and recognizable metaphases, 1 (patient 3) had the first cytogenetic analysis done in a recurrent tumor, 1 (patient 18) was not treated and 1 (patient 13) died after 3 months of causes not related to the tumor. Follow up in the remaining 15 patients was 12 to 53 months, with a mean of 31 months. None of these patients received intravesical chemotherapy before the first recurrence. At least 1 recurrence was noted in 5 of these 15 patients (table 4), 2 of which had cells with chromosomal numbers in the hyperploid range (table 5). In these non-infiltrating carcinomas progression occurred only once (patient 17) and the primary tumor showed a hyperploid chromosomal range. Patient 1 had a tumor with a normal karyotype and suffered 2 recurrences with a small grade 1 tumor.

Table 4
Recurrence and progression in the superficial tumors

Tumor Stage	Tumor Grade	Total No Patients	No. Pts. with Recurrence	No. Pts. with Progression	
				Stage	Grade
pTa	2	15	5	1	-
pT1	2	4	3	1	1
pT1	3	1	1	1	-
Totals		**20**	**9**	**3**	**1**

Of the 8 patients with stage pT1 tumors and recognizable metaphases 3 were not evaluable: 1 (patient 25) had nephroureterectomy, 1 (patient 22) died of a myocardial infarction and 1 (patient 24) had radiotherapy. Follow up in the remaining 5 patients was 4 to 49 months (mean 16 months). Four of these patients had a recurrence. Thus, 6 of the 9 patients with a recurrent tumor had hyperploid cells in the primary tumor.
The disease progressed in 4 patients with a superficial bladder tumor. All primary tumors had cells with chromosomal numbers in the hyperploid range but in 3 the modal number was hypodiploid or peridiploid. When the 8 evaluable patients with a primary superficial bladder tumor and a hyperploid chromosomal range are considered together, a recurrence was noted in 6 (4 with progression). Of the 12 evaluable patients with primary tumors in the hypodiploid or peridiploid range, 3 had a recurrence (none with progression).

Table 5
Recurrence and progression in relation to cytogenetic data at first examination

Pt.	Stage	Grade	Followup (months)	No. recur.	Mo.until first recur.	Progression	Chromosome range	Modal No.	Marker*
1	Ta(m)	2	38	2	16	-	46	46	-
11	Ta	2	40	2	7	-	36-46	44	-
12	Ta	2	25	1	17	-	42-44	43	-
16	Ta(m)	2	13	1	6	-	35-71	43/70	-
17	Ta(m)	2	53	4	10	T1/G2	38-78	43	-
19	T1	2	6	2	2	T1/G3 plus Ca in situ(m)	35-72	65	submedian, median and terminal
20	T1	2	49	1	3	-	28-73	53	subterminal
23	T1(m)	2	6	1	4	T2(m)G2	46-92	46	-
26	T1(m)	3	4	1	2	T3G3	23-80	46	-

* Unidentifiable structurally abnormal chromosome with the centromere in the submedian, median, subterminal or terminal region.
Mo : Months.
(m) : Multifocal.

Discussion

Cytogenetic analysis of solid tumors is difficult. The most important problem is the preparation of a good cell suspension. With bladder tumors the consistency of the tumor material appears to be relevant, as well as the amount of tissue available and the disaggregation technique used. Genuine papillary tumors that are of soft consistency can be worked easily into a cell suspension by mechanical means. However, it is almost impossible to make a good suspension by the direct technique with solid material as in our study. To reduce the risk of false negative karyotypes a great number of cells must be analyzed. However, when only a few chromosomally abnormal cells are found one may assume that these cells represent the malignant portion of the tissue when 2 or more cells show the same abnormalities. Mitoses were seen in most tumors but recognizable metaphases were noted in only 45 per cent. Technical improvements in cytogenetic studies are especially important for the low grade, low stage bladder cancers, for which

new objective criteria of malignant behavior are needed urgently. Near diploid tumors of all 3 pathological grades are reported to have a low proliferation rate.[6] Relatively more mitoses and recognizable metaphases were seen with the direct technique in invasive and grade 3 tumors. Nevertheless, mitoses were observed in 64 per cent of the non-invasive tumors, which implies, as is our recent experience, that with application of newer techniques[17-20] and growing experience the percentage of recognizable metaphases, also in superficial bladder tumors, will be higher than has been found to date.
In our study, as in those of others,[3-10] non-invasive tumors were mostly hypodiploid or diploid. Submucosal invasive tumors showed a wide range of modal numbers. Even 4 of the 17 deeper infiltrating tumors had a diploid mode. However, when a distinction is made between tumors with cells having chromosomal numbers of 49 or less and those with chromosomal numbers greater than 49, there is a difference. All invasive tumors, even if only submucosally, showed hyperploid cells. In accordance with previous reports[3,4,9] our grade 2 tumors showed a wide range in chromosomal mode, as well as in number of chromosomes in individual cells. Especially, the invasive grade 2 tumors had a wide range in chromosome number. With the criterion of hyperploidy (more than 49 chromosomes in cells) all invasive grade 2 tumors had cells with hyperploid numbers. Apparently, the range in chromosomal counts is a better reflection of invasion than the modal number. The wider the range of the chromosome number the more advanced the tumor is biologically. The interpretation would be that the tumors with a wide range of chromosomes show a much higher heterogeneity of tumor cells at a more advanced biological state than the hypodiploid or diploid tumors, or those with a sharp modal number. Therefore, such heterogeneity is reflected in the aggressive behavior of the tumor.

It is well known that invasive and non-invasive lesions occur in the large intermediate group of grade 2 tumors.[21] The need for tumor markers is greatest in these patients, since there also is less correlation between tumor morphology and progression. Studies on deoxyribonucleic acid (DNA) analysis have revealed a division that separates the grade 2 tumors as being 67 per cent diploid and 33 per cent grossly aneuploid.[22,23] Of the 74 grade 2 tumors 48 (65 per cent) were non-invasive and 26 (35 per cent) were infiltrating, as were almost all grade 3 tumors (table 1). The finding that non-infiltrating grade 2 tumors nearly always are hypodiploid or peridiploid, while infiltrating grade 2 bladder tumors always have hyperploid cells supports the idea that there is a distinction within the group of intermediate grade bladder tumors, cytogenetically and clinically.

Nearly all investigators have reported an increasing incidence of markers with increasing malignancy. Recurrence and progression rate among non-invasive and superficially invasive low grade bladder tumors with markers are reported to be much higher.[3,9,24] With our routine technique in non-banded preparations we found markers in many hypodiploid or near diploid tumors, and in most hyperploid cancers. No difference in the frequency of markers according to tumor stage and grade was found. Only 6 tumors were analyzed with banding techniques, including 4 of the 18 with marker chromosomes. Possibly, the techniques used in our study failed to detect subtle chromosomal abnormalities. The submedian marker was seen predominantly in non-invasive tumors. The subterminal and terminal markers occurred only in hyperploid cancers. To date few studies concerning banding in bladder tumors without longterm culture have been reported.[8,9,25-28] In all of these studies only a limited number of tumors were evaluated by banding. Developments

in chromosome banding techniques during the last years have indicated that karyotypic changes that cannot be established with routine methods often are revealed when the chromosomes are analyzed with various banding techniques in cultured cells.[17-19] With a direct method in cytogenetic analysis of solid tumors, a difficulty encountered especially in non-infiltrating tumors was the sparcity of analyzable metaphases. Moreover, the chromosomes frequently were fuzzy and contracted, and not suitable for banding. With our techniques cells with abnormal chromosomal counts or consitution may have been missed. However, long-term (more than 24 hours) culture is a more labor-intensive and time-consuming process. Also, it is not certain whether the cells growing in vitro represent the spectrum of cells present in the initial suspension. With a modified direct method in recent patients,[20] we nearly always can see metaphases of good quality, at least suitable for C-banding and many times also for G-banding.
The application of banding techniques allowed the identification of tumor-specific chromosomal rearrangements, such as t(9;22) in chronic myeloid leukemia,[29] t(8;14) in Burkitt's lymphoma,[30] del(13q) in retinoblastoma,[31] del(11p) in Wilms' tumor[32,33] and an isochromosome of the short arm of chromosome # 12,i(12p), in seminoma and malignant teratoma of the testis.[34,35] In bladder tumors non-random chromosomal changes have been demonstrated,[19] for instance an isochromosome of the short arm of chromosome # 5, monosomy of chromosome # 9 and loss of material from the short arm of chromosome # 11. This latter condition is of interest in view of the location of the c-Ha-ras oncogene.[36]

Only when banding techniques are applied in larger series of bladder tumors with more extensive follow up, it will be possible to determine whether specific structural chromosomal abnormalities can be used for diagnosis and prognosis in patients with bladder cancer. To date chromosomal analyis did not identify unequivocally the earliest irreversible changes predicting invasion and metastasis. Just as in light microscopy, electron microscopy[37] and the use of biochemical markers, such as ABH antigen deletion,[5] chromosomal analysis can identify only specific risk factors regarding the future behavior of bladder tumors. When present, these risk factors should be used together.

Nuclear DNA measurements have shown a correlation between the degree of ploidy and the degree of malignancy.[22,23] A general similarity between the results of chromosome and DNA analysis is reported.[10] However, DNA analysis cannot distinguish between diploid and near diploid tumors,[10] it cannot distinguish individual cells and it cannot establish specific morphological chromosomal abnormalities, which may recommend that cytogenetic examination should not be replaced by flowcytometry.

We conclude that the range in chromosomal counts appears to be a better reflection of invasion than the modal number. Noninvasive grade 2 tumors are mostly hypodiploid or diploid. Cytogenetically, there seems to be no difference between the invasive grade 2 and grade 3 tumors. During the limited follow up, recurrence was noted in hypodiploid, peridiploid and hyperploid tumors. Progression possibly is limited to tumors with hyperploid chromosomal numbers. Such tumors appeared to be more advanced biologically, even if they had a near diploid modal number. Application of new cytogenetic techniques, especially the advanced banding techniques, and evaluation during a longer follow up are needed to obtain more information about the prognostic meaning of marker chromosomes.

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Chapter III

Grading in superficial bladder cancer

1. Morphological criteria

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Summary

In a prospective study on grading of superficial papillary neoplasms of the bladder a distinction was made in tumors showing only increased cellularity without appreciable cellular and nuclear deviation (called grade 1), tumors showing slight cellular variation (grade 2a) and tumors showing clear cytologic deviation and a tendency to loose normal polarity (grade 2b). A total of 91 patients with a superficial tumor was evaluable for follow up (mean 24 months). Grade 2a tumors appeared to recur much later and in fewer patients than grade 2b tumours. Progression was seen in 4% of grade 2a tumors and in 33% of grade 2b carcinomas. Adapting our results to the W.H.O.-grading system we propose to classify as low grade all tumors in this study defined as grade 1 and 2a, and as intermediate grade tumors in this study defined as grade 2b.

Introduction

In the majority of patients with a first diagnosis of bladder cancer, the tumor shows no invasion or only invasion of the lamina propria (Cutler et al., 1982). Once diagnosed, progression and recurrence rate of these superficial tumors are found to be associated with different histopathological parameters, such as depth of invasion, grade, papillary or nonpapillary growth (Koss, 1975; Kern, 1984), number and size of visible tumors (Heney et al., 1983; Lutzeyer et al., 1982) and the presence of moderate to severe dysplasia in non-tumor bearing areas of the bladder (Althausen et al., 1976; Cutler et al., 1982). For prediction of the prognosis newer approaches such as deletion of blood group isoantigens (Summers et al., 1983), cytogenetic studies (Sandberg, 1977; Summers et al., 1983), DNA and RNA histograms (Devonec et al., 1981) and morphometric studies (Ooms et al., 1983) are available. Nevertheless, cytohistological grade is often the most important single item of prognostic value.

Several grading systems have been described (Broders, 1922; Bergkvist et al., 1965; Mostofi et al., 1973; Pugh, 1973; Koss, 1975; Friedell et al., 1976). They differ especially in the definition of grade 1 papillary lesions, and the variable interpretation of grade 2 neoplasms.

To analyse the prognostic significance of the different histopathological criteria, in this study the group of so called intermediate grade tumors were separated in two subgroups. The grading criteria used were correlated to tumor stage, recurrence rate and progression.

Patients and methods

Between 1980 and 1986 168 patients with a newly diagnosed transitional cell carcinoma (T.C.C.) of the urinary bladder were studied (see table 1). Patients were assessed according to the requirements of the T.N.M.-system (Harmer, 1978). At initial presentation urine was collected for cytologic examination, and biopsies were taken of the tumor and any suspected area, and of preselected areas with normal looking urothelium, distant form the tumor (lateral to each ureteral orifice and in the midline posteriorly). From every tumor multiple slides were graded by two pathologists, making one final classification. When more than one grade or stage was seen in tumor specimens of the same patient, or infiltration was doubtful, the tumor was classified according to the highest

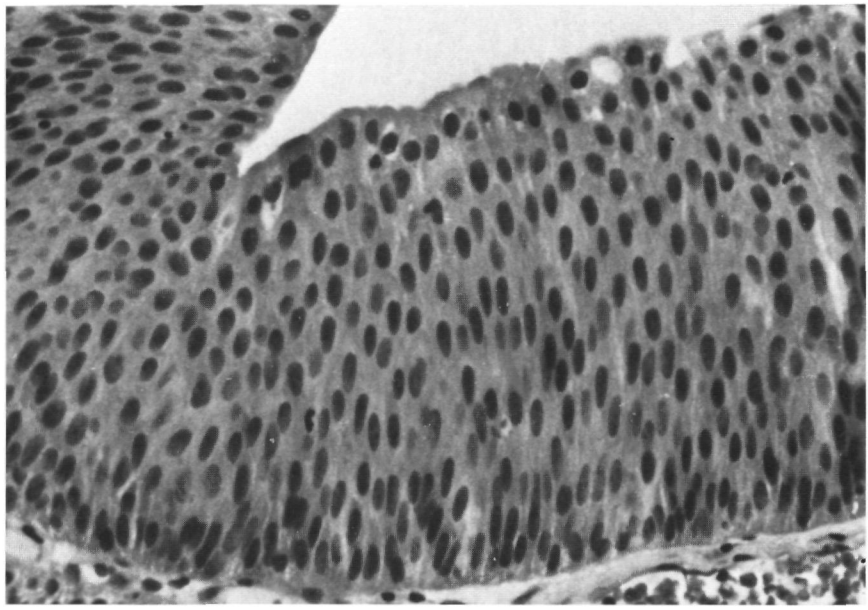

Fig. 1 Grade 1 papillary urothelial tumor. Slightly irregularly thickened epithelium. No appreciable cellular pleomorphism.
(HE 250x).

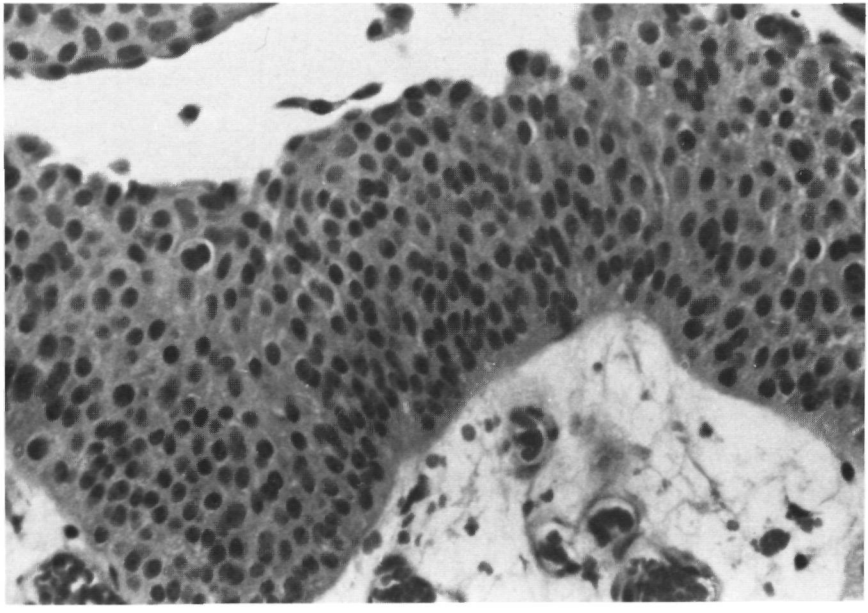

Fig. 2 Grade 2a papillary urothelial tumor. Slightly irregularly thickened epithelium with some cellular and nuclear deviations.
Normal polarity of nuclei. (HE 250x).

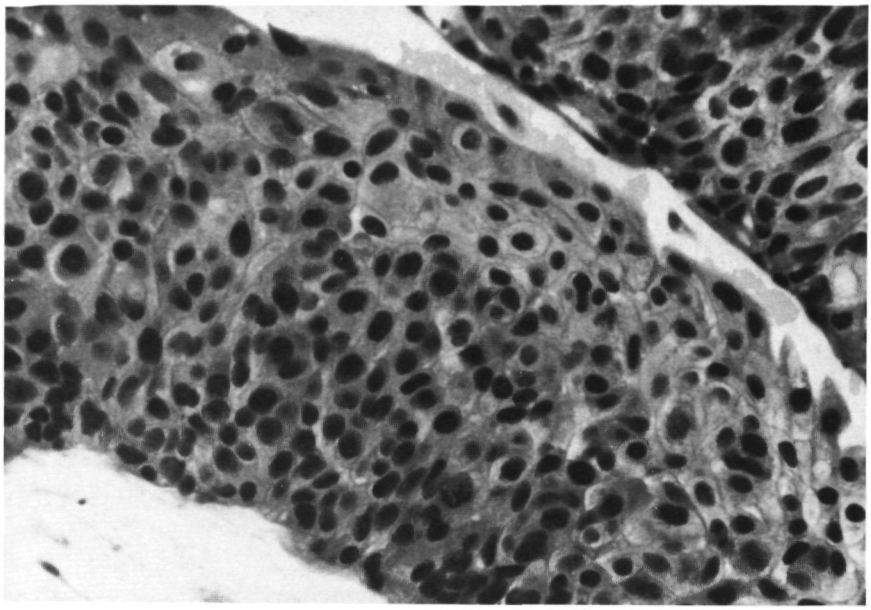

Fig. 3 Grade 2b papillary tumor. Irregularly thickened epithelium with loss of normal polarity of nuclei. Moderate abnormalities of cells and nuclei. (HE 250x).

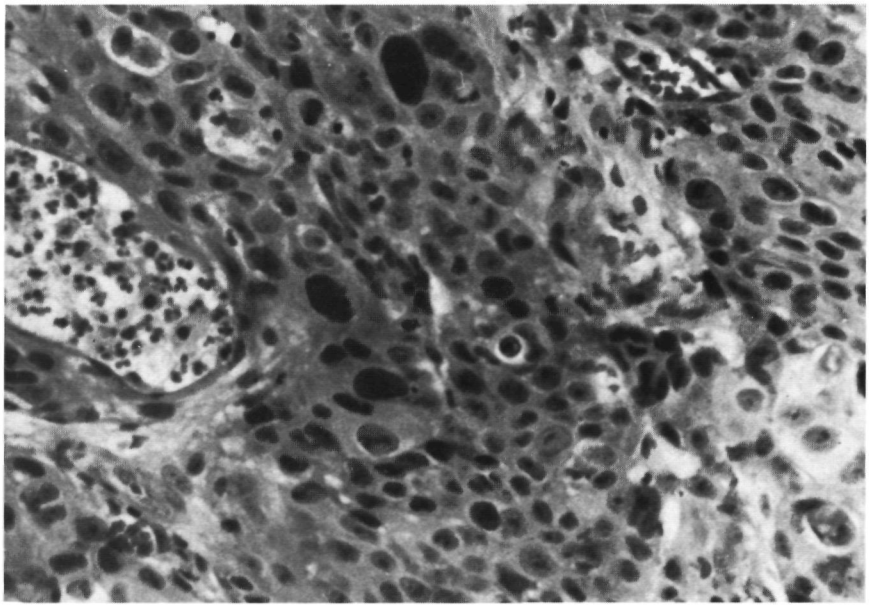

Fig. 4 Grade 3 papillary urothelial tumor. Irregularly thickened epithelium with total disarrangement of nuclei. Severe pleomorphism of cells and nuclei. (HE 250x).

grade and stage. If suspicious or malignant cells were found in the urine, cytology was considered positive. Pathological parameters at the time of the initial diagnosis were correlated with the clinical course in 122 patients with a superficial (Ta or T1) tumor, only treated by TUR and followed for 3 to 60 - mean 24 - months.
If chemotherapy, immunotherapy or radiotherapy was applied, or tumor progression was diagnosed, follow up ended.
The recurrence rate was defined as the total number of cystoscopies at which recurrences were observed and microscopically proven, divided by the sum of months of follow up till the last cystoscopy, multiplied by 100. For tumor progression, subseqent deeper invasion was taken as criterion. Statistical analyses were done with the chi-square test.

Grading system

Grade 1 (fig. 1) was applied to tumors, covered by slightly and irregularly thickened transitional epithelium which showed no appreciable cellular deviation (Bergkvist et al., 1965; Koss, 1975). Tumors covered by irregularly thickened transitional epithelium with slight cellular deviation and some variation in size of cells and nuclei were called grade 2a. Normal polarity of individual cells was seen here (fig. 2). In grade 2b tumors, (fig. 3) the epithelium was thickened and displayed distinct cellular irregularity with clear variation in nuclear and cellular size. Hyperchromasia was quite noticable. There was a tendency to loose the normal polarity of individual cells. Grade 3 tumors (fig. 4) were characterized by considerable cellular deviation with strong variation in size and shape of cells and nuclei. Individual cells and epithelial cords were disarranged. Locally there could be complete loss of transitional epithelial characteristics.
The inter- and intra-observer variability in grading was tested at the end of the study. Randomly chosen slides from 20 patients were separately seen at two occasions by two pathologists. In a last common session the slides were reevaluated to make one final classification.

Results

Tumor stage and grade

Tumors classified as grade 1 were never invasive (see table 1). Grade 2a carcinomas appeared mostly noninvasive, grade 2b in the majority submucosally or deeply invasive ($p=0.001$). Grade 3 tumors were significantly deeper infiltrating than the grade 2b cases ($p=0.004$).
Of 89 new cases with a superficial tumor where selected mucosal biopsies of normal looking urothelium were examined, only in 4 severe dysplasia was shown (see table 2). In 3 of these cases this regarded patients with a multifocal T1 tumor. No correlation was demonstrable between the occurrence of slight dysplasia in the selected mucosal biopsies, and the clinical course.
Cytology of prae-cystoscopy urine was positive in 13 of 57 cases with a grade 2a tumor (23%), in 26 out of 40 cases with a superficial grade 2b tumor (65%).

Clinical course of superficial (pTa - pT1) tumours

91 Patients with a superficial tumor were evaluable for follow up, from 3 to 60 (mean 24) months (see table 3). The clinical behavior of grade 1 tumors was not significantly different from grade 2a carcinomas. Of the 54 evaluable grade 2a patients, 42 (78%) remained disease-free, compared with only 9 (43%) of the 21 grade 2b tumors ($p = 0.008$)

Table 1
Correlation between tumor stage and grade

Grade	Stage						
	Ta	T1	T2	T3	T4	*Tis(m)	Totals
1	13	-	-	-	-	-	13
2a	54	3	-	-	-	-	57
2b	14	30	5	9	1	-	59
3	-	8	11	15	3	2	39
Totals	**81**	**41**	**16**	**24**	**4**	**2**	**168**

*Tis(m): Carcinoma in situ on multiple places.

Table 2
Histology of selected mucosal biopsies of normal looking urothelium in new cases with superficial tumors

Grade	No. of Patients	Histological appearance				
		Normal	Hyperplasia	Mild Dysplasia	Severe Dysplasia	Carcinoma
G1	9	8	0	1	0	0
G2a	45	38	0	7	0	0
G2b	31	26	0	2	3	0
G3	4	3	1*	0	1*	0
Totals	**89**	**75**	**1**	**10**	**4**	**0**

* Both in the same patient.

Table 3
Relation of tumor grade and stage with clinical course for the superficial tumors

	Grade				Stage	
	1	2a	2b	3	Ta	T1
n patients evaluable	12	54	21	4	71	20
n patient disease free	8(67%)	42(78%)	9(43%)	2(50%)	50(70%)	11(55%)
recurrence rate	2.3	1.5	3.1	4.3	2.1	3.0
tumour progression	1(8%)	2(4%)	7(33%)	1(25%)	4(6%)	6(30%)

Follow up 3 to 60 - mean 24 - months

Table 4
Recurrence probabilities
(Kaplan-Meier method)

Stage-Grade	Years of follow up		
	1	2	3
TaG2a	0.05	0.13	0.43
TaG2b	0.31	0.65	0.83
T1G2b	0.49	-*	-*

-* Not evaluable because of chemotherapy or tumor progression.

There is a clear difference in recurrence rate between grade 2a and grade 2b tumors, respectively 1.5 and 3.1. The observed 1-year recurrence probabilities were 5, 31 and 49 per cent, respectively, for TaG2a, TaG2b and T1G2b tumors (see table 4). These probabilities increased after three years to 43 per cent for TaG2a tumors, 83 per cent for TaG2b.

Tumor progression was seen in 2 of the 54 grade 2a tumors (4%). Both being Ta at first diagnosis, recurred as a T1 tumor, one after 13 months, the second after 53 months. Of the 21 grade 2b carcinomas 7 showed tumor progression (33%): 2 out of 8 TaG2b tumors, 5 out of 13 T1G2b tumors.

Five recurred as deeply infiltrating bladder cancers, four of which half a year after the initial diagnosis. So progression was significantly more frequently seen with grade 2b than with grade 2a tumors ($p = 0.002$).

Multiple tumors were seen in 2 of the 12 evaluable patients with a grade 1 lesion (17%), 10 of 54 with a grade 2a (18%), and 4 of the 21 (19%) with a grade 2b carcinoma. Multiplicity could not be seen as an explanation for the difference in clinical behavior between grade 1 - 2a, and grade 2b tumors.

Table 5

Inter- and intra-observer variability in grading

Case number	Original Classification	Testing Session				Final classification Pathologist I+II
		Pathologist I		Pathologist II		
		1	2	1	2	
1	1	2a	2a	2a	2a	2a
2	1	2a	2a	2a	2a	2a
3	1	2a	2b	2a	2a	2a
4	2a	2a	2b	2b	2b	2b
5	2a	2a	2a	2a	2a	2a
6	2a	2a	2a	1	2a	2a
7	2a	2b	2a	2a	2a	2a
8	2a	2b	2b	2b	2b	2b
9	2a	2b	2b	2b	2b	2b
10	2b	3	3	3	3	3
11	2b	3	2b	3	2b	3
12	2b	2b	3	2b	2b	2b
13	2b	3	3	2b	2b	2b
14	2b	3	3	3	3	3
15	2b	3	3	2b	3	3
16	2b	2b	2b	2b	2b	2b
17	3	3	3	2b	2b	2b
18	3	3	3	3	3	3
19	3	3	3	3	3	3
20	3	3	3	3	3	3

Inter- and intra-observer variability in grading

Three cases, originally classified as grade 1, finally were graded 2a (see table 5). In four cases the original classification was grade 2b, the final grade 3. So fairly often the distinction made between grade 1 and grade 2a tumors respectively between grade 2b and grade 3 tumors appeared not consistent. Of more importance is that three cases originally graded 2a, finally were graded 2b. Common practice in our clinic is, when a tumor recurrence or metastasis is diagnosed, to reexamine in a common session from pathologists and urologists all slides from preceding tumors, comparing the tumor grade formerly given with the then known clinical behavior and eventual additional parameters. With respect to the distinction between grade 2a and 2b tumors, much less discrepancy was seen between the testing sessions at the end of the study.

Discussion

Difficulties in the comparison of grading in publications on bladder tumors exist especially in superficial papillary tumors. For some pathologists a grade 1 papillary lesion shows only thickened urothelium without significant nuclear abnormalities (Bergkvist et al., 1965; Koss, 1975). For others grade 1 applies to tumors that have the "least degree of cellular anaplasia compatible with a diagnosis of malignancy" (Mostofi et al., 1973) or nuclear pleomorphism and disturbance of cellular architecture (Friedell et al., 1976). Even when the same grading system is used, there appears to be a disturbingly high intra- and interindividual inconsistency in tumor grading (Ooms et al., 1983). Aim of this study was the search for a sharper histological distinction between low and intermediate grade urothelial cell carcinomas based on the clinical behavior of these tumors.

Almost all grade 1 and grade 2a tumors appeared to be stage Ta. There was a close correlation between grade 2b and infiltration. Clinical behavior of TaG2b tumors appeared worse than for TaG2a tumors. Therefore grading is on its own of prognostic value apart from tumor stage.

We found a striking association between tumor grade and positive urine cytology, like is reported before (Heney et al., 1983). In patients with newly diagnosed noninfiltrating (Ta) tumors, abnormalities in selected mucosal biopsies were seldom found, mostly only slight, and if so, the clinical course was not significantly different from those with normal mucosal biopsies, as is shown before (Heney et al., 1983). We agree with Wallace and associates that there is no good case for routine mucosal biopsies in such patients on first presentation (Wallace et al., 1979).

With regard to recurrences and progression, we did not find a significant difference between grade 1 and grade 2a tumors. There was however a very significant difference in recurrence probability and the rate at which tumors recurred between Ta grade 2a and 2b tumors, after one year 5 and 31 per cent respectively. Similarly striking was the difference in tumor progression between grade 2a and grade 2b tumors, 4 and 33 per cent respectively, occurring late with grade 2a carcinomas, fairly early with grade 2b lesions. These findings are in accordance with the conclusions of others that with increasing tumor grade time intervals from presentation to recurrence and progression become shorter (England et al., 1981; Cutler et al., 1982).

In our study too there was some inconsistency in grading between the two pathologists at various testing sessions. There were however only minor differences in grading between the testing sessions at the end of the study, and the final common classification.

Apparently through the years our pathologists learned to handle the grading criteria used. Because of the importance of histopathological grading a continuing study is necessary, ever again comparing the primary slides of first diagnosis with biopsy specimens from recurrences.

With respect to recurrences and progression according to tumor stage this series appeared to be in accordance with other studies (Greene et al., 1973; England et al., 1981; Cutler et al., 1982). Eight per cent of our cases were classified as being grade 1. This low percentage is due to the strict adherence to the grading criteria used. Pooling our 13 grade 1 and 57 grade 2a tumors in one group, the percentage for low grade tumors in our material is 42, more in accordance with the percentage of low grade tumors in other series, varying between 23 per cent (Friedell et al., 1976) till 35 per cent (Cutler et al., 1982).

Mostofi (1975) stated: basically there are two categories of bladder tumors, grade I and grade III. As we demonstrated in our prospective study the intermediate category can indeed be separated in two groups on cytohistological and clinical grounds: one, grade 2a, belonging to grade 1; the other, grade 2b, resembling grade 3. We found a difference between grade 2a and grade 2b tumors with respect to chromosomal numbers (Pauwels et al., 1987; this thesis chapter IV). Clearly grade 1 tumors in the W.H.O.-grading system ought to be defined as our grade 1 and grade 2a tumors.

It is obvious that classifying superficial bladder tumors in two groups with a rather distinct prognosis has far going consequences regarding mode of follow up and the application of prophylactic chemotherapy. For patients with a solitary noninfiltrating low grade tumor the first cystoscopic control could be postponed till at least half a year after the initial diagnosis. Application of intravesical chemotherapy could be reserved for those patients showing multifocal disease, or recurrences, and should be done with those tumors showing histological clear or strong deviations, our grade 2b and grade 3 lesions.

In summary we conclude that the interpretation of the W.H.O.grading classification should be that in papillary urothelial cell carcinomas of the bladder those tumors should be called low grade which show irregularly thickened epithelium with or without slight nuclear and cellular deviations, but with normal polarity of individual cells, in this study called grade 1 and grade 2a. The classification grade 2 should be given to tumors defined by us as grade 2b. Grade 3 applies to tumors with the most severe degree of cellular anaplasia.

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Chapter IV

Grading in superficial bladder cancer.

2. Cytogenetic classification.

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Summary

Cytogenetic analysis was performed in 92 newly diagnosed transitional cell bladder carcinomas. The results of this analysis were correlated with the clinical course in the superficial tumors. Low grade tumors appeared to have hypo- or peridiploid chromosomal numbers. Intermediate grade tumors were characterized by chromosomal counts up to the hyperdiploid range, but could have a peridiploid modal number. With respect to the clinical course of superficial tumors the range in chromosomal counts appeared to be more reliable than the modal number. The significant difference in chromosome numbers between low, intermediate and high grade tumors may be considered as a biological basis for grading.

Introduction

The natural history of a papillary tumor of the bladder is unpredictable. Next to tumor stage, grade is at present the most important indicator of the clinical behavior of a bladder tumor (Kern, 1984). However, reports regarding grade are not always completely comparable because different grading systems are used, or different interpretations are given to one grading system (Hofstädter et al., 1984). Chromosomal analysis is mentioned as one of the more objective criteria of the malignant potential of bladder cancer (Lamb, 1967; Spooner and Cooper, 1972).

Cytogenetic studies showed a positive correlation between histological grade and modal chromosome numbers for low and high grade bladder tumors (Spooner and Cooper, 1972; Wijkström et al., 1984). In intermediate grade tumors normal as well as abnormal modal numbers were found. In another study (Pauwels et al., 1987; this theses chapter III) an interpretation of the W.H.O. grading criteria was established which appeared to correlate with rate of recurrences and tumor progression.

The aim of this study was to investigate whether this interpretation of the W.H.O. grading classification could be confirmed by a cytogenetic study.

Patients and methods

Between 1980 and 1986, from 92 patients with a primary transitional cell carcinoma of the bladder only treated by TUR,chromosomal data from tumor specimens were achieved (see table 1).

Table 1.
Number of patients in relation to tumor stage and grade.

Grade	Tumor stage			Totals
	Ta	T1	T2-4	
G1	31	1	–	32
G2	9	15	11	35
G3	–	5	20	25
Total	**40**	**21**	**31**	**92**

Staging was done according to the T.N.M.-system (Harmer, 1978). As grade 1 were classified tumors showing increased cellularity with or without slight nuclear and cellular variations but with normal polarity of individual cells. Tumors showing clear cytological deviations with a tendency to loose normal polarity were classified grade 2. Tumors were called grade 3 if considerable cellular deviation was seen, with strong variations in size and shape of cells and nuclei and a loss of normal cellular architecture.
Patients with a superficial (Ta-T1) low or intermediate grade tumor were followed by cystoscopy for 3 to 62 - mean 28 - months. If chemotherapy, immunotherapy or radical therapy was applied, or tumor progression occurred, follow-up ended.

Cytogenetic analysis.

For microscopic analysis of chromosomes, cells in metaphase were obtained from a suspension of single cells. After colcemid arrest, hypotonic treatment and fixation, the chromosomes were routinely stained with Giemsa. For further identification of chromosomes, specific banding techniques are available. The techniques used for cytogenetic analysis -direct method or short term culture - have been described previously (Smeets et al., 1985).
Analysable metaphases, at least 5 (average 28) per case were photographed and karyotyped according to the Paris nomenclature (I.S.C.N., 1978). The tumors were classified according to the modal chromosome number and chromosome range. The latter classification distinguished the tumors with cells only in the hypodiploid or peridiploid range (49 or less) and cells with more than 49 chromosomes (hyperdiploid).
Marker chromosomes, structurally abnormal chromosomes, as mentioned in this study were shown by routine techniques, mostly in non-banded preparations.

Results

Normal bladder tissue.

In 15 patients with non-infiltrating tumors, random biopsies from cystoscopically normal bladder tissue showed neither histopathological nor cytogenetic abnormalities.

Tumor stage and grade.

The majority of non-invasive tumors (Ta) had peridiploid chromosomal numbers (see table 2).

Table 2.
Tumor stage in relation to chromosomal numbers.

chromosomal number	Number and stage of tumors		
	Ta	T1	T2-4
modal number ≤ 49	35	9	6
modal number > 49	5	12	25
range ≤ 49	31	–	–
range > 49	9	21	31

In all infiltrating tumors, cells with more than 49 chromosomes were present, although the modal number often was peridiploid. The difference in the range of chromosomal numbers between non-invasive and invasive tumors is significant ($p < 0.001$).

As is shown in table 3 all except one grade 1 tumors had a modal number of 49 or less. In nearly half of the 35 grade 2 tumors the modal number was peridiploid. Nearly always, however, cells in the hyperdiploid range were seen like in all grade 3 tumors. The differences in chromosomal numbers (mode and range) between grade 1 and grade 2 tumors are significant ($p < 0.001$). Between grade 2 and grade 3 tumors there was a significant difference in modal number ($p < 0.002$).

Table 3.
Tumor grade in relation to chromosomal numbers.

Chromosomal number		Number and grade of tumors		
		G1	G2	G3
modal number	≤ 49	31	17	2
modal number	> 49	1	18	23
range	≤ 49	28	3	–
range	> 49	4	32	25

Marker chromosomes (see fig. 1) were found in 17 of the 40 non-invasive tumors (43%) and in 31 of the 52 invasive cases (60%) ($p = 0.22$). Nine out of 32 grade 1 tumors showed markers (28%) compared with 39 of the 60 grade 2 and grade 3 cases (65%). So, in higher grade tumors more often marker chromosomes were found ($p = 0.003$).

Follow up of superficial low and intermediate grade tumors.

Fourty-two patients with a superficial low or intermediate grade tumor were evaluable for follow up (see table 4).

Table 4.
Correlation of results of chromosomal analysis with clinical course for superficial (Ta - T1) low and intermediate grade bladder tumors.

patients	Results of chromosomal analysis.					
	modal number		chromosomal range		marker chromosomes	
	≤ 49	> 49	≤ 49	> 49	no	yes
evaluable	37	5	28	14	24	18
disease free	19 (51%)	0[a]	16 (57%)	3 (21%)[b]	13 (54%)	6 (33%)[c]
with tumor progression	7 (19%)	2 (40%)[d]	3 (11%)	6 (43%)[e]	4 (17%)	5 (28%)[f]
recurrence rate	3.0	2.7	2.9	3.0	2.2	3.9

Follow up 3 to 62 - mean 28 - months.
Recurrence rate: n recurrences/n months patient follow up x 100
[a] $p = 0.07$; [b] $p = 0.16$; [c] $p = 0.50$; [d] $p = 0.62$;
[e] $p = 0.05$; [f] $p = 0.63$

Fifty-one percent of the tumors with a peridiploid modal number did not recur. In the group with hyperdiploid modal numbers all tumors recurred ($p = 0.07$). With respect to tumor progression no significant difference was seen between these two groups ($p = 0.62$). If, however, in a tumor chromosomal numbers in the hyperdiploid range were shown, tumor progression was seen significantly more often than in the group with lower

chromosomal numbers ($p = 0.05$).
The tumor recurrence rates showed no significant differences between the groups. However, estimations using the Kaplan-Meier method show that in the group with a hypo- or peridiploid chromosomal range the first 18 months only 10% had a recurrence, which figure rose to 69% after 42 months. In the group with a hyperdiploid chromosomal range, 54% had at least one recurrence after 18 months. So, tumors with a hypo- or peridiploid chromosomal range clearly recurred much later.
In four cases progression was seen in tumors with near normal modal numbers but a hyperdiploid chromosomal range. With respect to the clinical course, no significant difference between the groups with or without marker chromosomes was observed.
An addendum shows the histopathological and cytogenetic data, and follow-up of the clinical course for each patient.

Discussion

In a previous study about grading in superficial bladder cancer, a clear correlation between morphological criteria and the clinical course of bladder tumors was found (Pauwels et al., 1987). The present study was done to see whether a correlation between chromosomal abnormalities and the grading criteria used could be demonstrated.
Grade 1 tumors appeared to have hypo- or peridiploid chromosomal numbers. Grade 2 tumors show in half of the cases a peridiploid modal number but nearly always they contain a number of cells with more than 49 chromosomes. In grade 3 tumors the chromosomal mode is strongly hyperdiploid. Other authors also reported that grade 2 tumors are heterogeneous with respect to modal number (Spooner and Cooper, 1972; Wijkström et al., 1984). The significant difference in chromosomal range between grade 1 and grade 2 tumors is not reported before.
Tumors graded by us as intermediate recur significantly earlier, and progress more often than low grade tumors (Pauwels et al., 1987). These grade 2 tumors appear to have chromosomal numbers up to the hyperdiploid range. This means that nuclear pleomorphism, enlargement and hyperchromasia probably reflect the increase in chromosome number and the increased aggressiveness of a tumor.
Tumors having a peridiploid modal number but a range till in the hyperdiploid region, tended to behave worse than tumors with only near normal chromosomal numbers. The finding of hyperdiploid modal numbers in intermediate grade superficial bladder tumors, like by FCM analysis higher DNA values, is a bad prognostic sign (Wijkström et al., 1984). However, a near normal modal chromosomal number does not always indicate a benign behavior of the tumor.
Marker chromosomes in superficial bladder cancers were regarded as a very bad prognostic factor (Falor and Ward, 1978; Summers et al., 1981). In our study this could not be confirmed. For establishing structural abnormalities so called chromosome banding techniques are necessary.
By the use of different banding techniques non-random chromosomal changes have been demonstrated in bladder tumors (Gibas et al., 1984; Atkin and Baker, 1985). The prognostic significance of the different changes are not yet known. The increasing variation in chromosomal number during the progression of the neoplasia is believed to confer proliferative advantage to the tumor cells (Yunis, 1983).
Our grading criteria in superficial bladder cancer were proved to have a clinical basis with

respect to tumor stage, rate of recurrences and tumor progression (Pauwels et al., 1987). The conclusion of this study may be that with chromosomal analysis we were able to give a biological meaning to our grading system.

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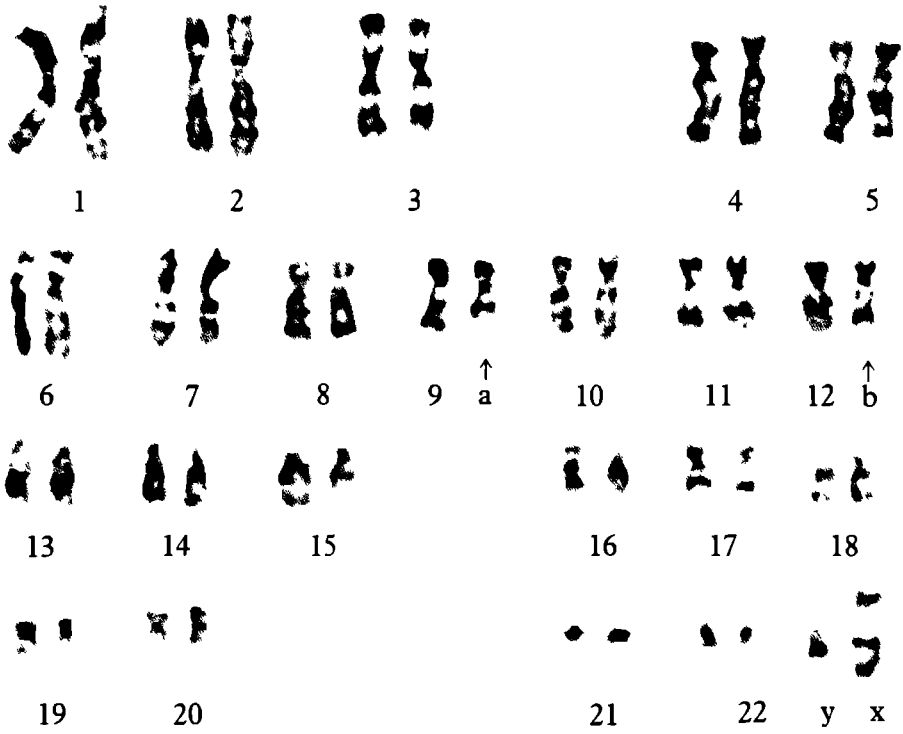

Fig. 1 A G-banded karyotype of a Ta Tumor.
The abnormal chromosomes are indicated by arrows; "a" has a deletion of the long arm (9 q–); "b" is an unrecognizable chromosome (marker chromosome).
(Giemsa, x 4000).

Addendum:

Histopathologic and cytogenetic data for the initial biopsy from individual bladder carcinoma patients with follow up.

Patient			Tumor		Chromosomes								Follow up	
								Ploidy[a]						
Case	Age	Sex	T	G	Range	Mode	n	2n	3n	4n	5n	Mar	n Recurr.[b] n Months	Progression
1	66	M	a(m)	1	19-46	46	5	21				–	1/16	+ (T2G3)
2	51	M	a(m)	1	40-45	43		40				+	3/41	–
3	27	M	a	1	45-46	46		5				–	–/26	–
4	63	M	a	1	34-48	46		7				–	–/38	–
5	64	M	a	1	40-90	43		19	1	5		+	–/30	–
6	65	M	a(m)	1	35-71	43/70		8	8			–	1/29	–
7	66	M	a	1	30-46	43		10				–	–/12	–
8	72	F	a	1	19-39	30	2	3				–	1/51	–
9	70	M	a	1	40-47	46		20				–	1/ 4	–
10	58	F	a(m)	1	40-45	43		16				–	2/16	+ (T1G3)
11	57	M	a(m)	1	38-78	43		19	3			+	3/53	+ (T1G1)
12	41	M	a(m)	1	45-46	45		5				+	4/24	–
13	59	M	a(m)	1	40-44	43		5				–	–/30	–
14	63	M	a	1	41-47	43		16				–	–/12	–
15	73	M	a	1	46-48	48		8				–	–/ 3	–
16	55	M	a	1	43-46	44		7				–	1/30	–
17	17	M	a	1	45-46	46		5				–	1/48	–
18	77	M	a	1	42-47	46		11				–	1/21	–
19	56	M	a	1	35-45	42		10				+	chemotherapy	
20	60	F	a	1	32-49	46	10	74				+	–/42	–
21	58	M	a	1	34-46	46		10				–	–/52	–
22	91	M	a(m)	1	38-46	46		46				–	–/12	–
23	65	M	a	1	46	46		5				–	–/54	–
24	56	M	a	1	36-46	44		13				–	3/48	–
25	71	M	a	1	35-48	46		5				+	–/42	–
26	61	M	a(m)	1	30-46	40		5				–	2/66	–
27	63	M	a	1	42-44	43		5				–	1/36	–
28	60	M	a	1	44-46	46		10				–	–/12	–
29	76	M	a	1	40-45	45		9				–	chemotherapy	
30	48	M	a	1	37-46	45		15				–	–/12	–
31	66	M	a	1	36-47	45		16				+	–/15	–
32	65	M	a	2	19-45	42	11	16				+	4/24	+ (T1G2)
33	73	M	a	2	60-89	70			7			+	1/28	+ (T2G3)
34	82	M	a	2	50-90	67		2	7	4		+	not fit for treatment	
35	61	M	a	2	50-75	64			20			+	1/60	–
36	72	F	a	2	38-57	45		11				+	–/18	–
37	66	M	a	2	23-44	40	4	31				+	1/26	–
38	78	M	a	2	39-87	45		31	1	2		+	1/25	–
39	44	M	a	2	36-49	46		27				+	chemotherapy	
40	83	M	a	2	40-83	78		13	13	1		+	chemothearpy	
41	55	M	1	1	37-72	44		47	1			+	1/20	–
42	78	F	1(m)	2	46-92	46		38	6	4		–	1/ 5	+ (T2(m)G2)
43	66	M	1(m)	2	35-72	65		4	7			+	1/ 4	+ (T2(m)G3)
44	77	M	1	2	28-73	53	2	9	5			+	1/62	–
45	86	M	1	2	46-120	70		14	53	2		+	death unrelated to cancer	
46	59	M	1	2	35-72	42		38	2			+	–/40	–
47	72	M	1	2	62-77	72			25			+	chemotherapy	

Patient			Tumor		Chromosomes								Follow up	
							Ploidy[a]							
Case	Age	Sex	T	G	Range	Mode	n	2n	3n	4n	5n	Mar	n Recurr.[b] n Months	Progression
48	53	M	1	2	35-57	43		13				+	radiotherapy	
49	54	M	1	2	19-69	44	11	38	6			+	death unrelated to cancer	
50	62	F	1	2	44-89	87		2	1	16		+	chemotherapy	
51	86	F	1	2	75-86	80			16	4		+	chemotherapy	
52	70	F	1	2	43-70	44		18	6			+	chemotherapy	
53	74	M	1	2	44-86	47		21		1		–	chemotherapy	
54	81	M	1	2	75-88	84				18		–	chemotherapy	
55	69	F	1(m)	2	23-80	45	4	7	4			–	1/ 4	+ (T3G3)
56	69	M	1	2	30-87	46		32	1	2		+	1/ 8	+ (T2G3M1)
57	73	F	1	3	70-89	73			3	3		+	radiotherapy	
58	68	M	1	3	86-100	90			5			–	radiotherapy	
59	45	M	1	3	65-90	70			5	2		–	–/ 9	–
60	60	M	1	3	43-100	88		1	1	13		–	1/ 3	–
61	65	M	1	3	43-147	146		1	1	1	4	+	1/ 5	–
62	73	M	2	2	43-75	46		27	6			–	–/24	–
63	75	M	2(m)	2	59-93	85			22	28		+	cystectomy	
64	84	M	2	2	30-89	72	1	2	15	2		+	radiotherapy	+ cystectomy
65	54	M	2	3	65-73	72			6	8		+	radiotherapy	+ cystectomy
66	70	M	2	3	53-76	73		2	23			+	radiotherapy	+ cystectomy
67	74	F	2	3	46-110	98		3	1	16	1	–	death unrelated to cancer	
68	82	M	2	3	19-93	58	3	3	3			+	radiotherapy	+ cystectomy
69	53	M	2	3	54-117	60		2	8		3	+	cystectomy	
70	71	F	2	3	42-70	44		5	1			–	radiotherapy	+ cystectomy
71	87	F	2	3	50-220	110				15	10	+	radiotherapy	
72	56	M	2	3	46-90	46/90		3	1	4		–	radiotherapy	+ cystectomy
73	70	F	2(m)	3	36-90	69		17	2	10		+	radiotherapy	
74	76	M	2	3	30-69	45/90		20	–	24		–	death unrelated to cancer	
75	67	F	3	2	53-81	77		2	28	1		+	cystectomy	
76	66	M	3	2	46-100	69		1	3	1		–	radiotherapy	
77	72	M	3(m)	2	40-69	46		4	2			+	cystectomy	
78	78	M	3	2	65-80	73			13			+	radiotherapy	
79	73	M	3	2	42-62	46		9	1			–	radiotherapy	
80	80	M	3	2	40-90	46/90		10		10		–	death unrelated to cancer	
81	69	M	3	2	46-90	46		6		2		–	cystectomy	
82	64	M	3	3	45-75	75		4	6			+	cystectomy	
83	74	M	3(m)	3	62-140	73			37		2	+	radiotherapy	
84	75	M	3	3	90-95	92				5		+	radiotherapy	
85	73	M	3	3	42-90	45		3	2			–	cystectomy	
86	56	M	3(m)	3	64-90	75			5	5		+	radiotherapy	
87	74	F	3	3	50-90	70			4	1		+	radiotherapy	
88	64	M	3	3	19-69	50	1	4	4	13		–	death of cancer	
89	70	M	3(m)	3	73-121	115			3	2	35	+	radiotherapy	
90	60	M	4(m)	2	40-140	43		19	4	1	5	–	radiotherapy	
91	66	M	4	3	65-135	69			13	1	6	–	death of cancer	
92	44	F	4	3	46-120	56		8	3	1		–	radiotherapy	

[a] Ploidy: n = 23± 11; 2n = 46 ± 11; 3n = 69 ± 11 etc.

[b] n Recurr. : number of recurrences per total number months of follow up.
n Months

Chapter V

Chromosomal analysis of bladder cancer. III.

Nonrandom alterations.

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Abstract

Chromosome analysis using G- and C-banding was performed in thirteen primary transitional cell carcinomas of the bladder. The chromosome preparations were obtained by a direct method. In eight tumors with a (near) diploid modal chromosome number the most frequently observed chromosome aberrations were: (partial) monosomy chromosome # 9 in four cases, deletion of chromosome # 10q in two cases and partial trisomy of chromosome # 1 in two cases.
In five tumors with a modal chromosome number in the triploid or tetraploid range the chromosomes # 1, # 3, # 7, # 9, # 11 and # 17 were numerically and or structurally abnormal in at least four cases. In three out of ten males, the Y-chromosome was missing. These findings suggest that the loss of chromosome # 9, and possible also loss of 10q is a primary event in the karyotypic evolution of transitional cell carcinoma of the bladder.

Introduction

Chromosome analysis of transitional cell carcinoma (TCC) of the urinary bladder has revealed the involvement of a number of chromosomes. In one study, the presence of an isochromosome i(5p), an isochromosome i(8q), deletion and translocation of chromosome # 8, monosomy # 9 and interstitial deletion of # 13 were noticed [1].
Another study reported the involvement of chromosomes # 1 and # 11 and to a lesser degree chromosomes # 3 and # 17 [2]. Also alterations of chromosomes # 6, and # 13 have been published as non-random abnormalities [3]. Recently deletions of 10q24 and 21q22, trisomy # 7 and aberrations of # 9, # 13, # 15 and # 20 were added to the list of alterations [4].
Hitherto, some dozens of cases are described with successful banding of the tumor chromosomes
After the introduction of a new technique in our laboratory, G-banded chromosomes could be obtained much more frequently during the past [5]. In this report the chromosomal findings of 9 cases, studied with this method, are combined with the results of four patients studied at an earlier date.

Materials and methods

The tumor samples were collected and transported in 0.5% sodium citrate + 0.5 μg colcemid/ml and prepared as described elsewhere [5].
All tumors were primary tumors. None of the patients, before first resection, received chemotherapy or radiation therapy. Two of them recurred. Further data on the 13 cases are shown in Table 1.
Clinical staging of the tumors was done according to the rules of the Union International Contre le Cancer [6] and grading according to the WHO system [7]. In addition, intermediate tumors were divided in G2a and G2b. The latter is less differentiated than the former [8].
In addition to the cells with G- and C-banded chromosomes, a number of unbanded metaphases could be analyzed. Therefore the chromosomes of at least 15 metaphases of every tumor were counted in order to increase the reliability of the chromosome number.

Results

The tumors fell into two groups: those with near diploid modal chromosome numbers (cases 1-8) and those with near tri- or tetraploid modal numbers.

Case 1.: Mode 46. Range 43-90.
Two G-banded and two C-banded metaphases with 46 chromosomes were analyzed. A 10q- [del(10)(q22)] was present. There were some (near) tetraploid cells, which were unsuitable for chromosomal analysis.
The modal karyotype was: 46,XY, del(10)(q22).

Case 2.: Mode 46.
All cells counted showed 46 chromosomes.
Two G-banded and two C-banded metaphases were analyzed. One chromosome # 9 showed a deletion of the long arm 9q-[del(9)(q22?)]. One chromosome # 12 was missing and one marker chromosome, probably derived from a chromosome # 12, was present.
The modal karyotype was: 46, XY, 9q-, -12, + mar.

Case 3.: Mode 45. Range 44-45.
Five G-banded and two C-banded metaphases were analyzed. Consistent features were monosomy # 9 and 10q-[del(10)(q22)].
The modal karyotype was: 45,XY, -9, del(10)(q22).

Case 4.: Mode 47.
All cells, except one, had 47 chromosomes.
Seven G-banded and two C-banded metaphases were analyzed. Consistent features were 2q-, 9q+, +i(8q). Furthermore, the short arm of one chromosome # 8 was probably abnormal.
The single diploid cell showed no karyotypic abnormalities.
The modal karyotype was: 47,XY, 2q-, +i(8q), 9q+.

Case 5.: Mode 46. Range 42-46.
Seven G-banded and two C-banded metaphases were analyzed. Consistent features were an extra chromosome 1p-[del(1)(p22)] and a missing Y-chromosome.
The modal karyotype was: 46,X, -Y, +1p-.

Case 6.: Mode 44. Range 41-72.
Three G-banded and two C-banded metaphases were analyzed. Missing were one copy of # 9, # 11, # 14 and the Y-chromosome.
Two structurally abnormal chromosomes were present: 1p-[probably del(1)(p13)] and an 11p+ chromosome.
There were some (near) triploid cells, which were unsuitable for chromosomal analysis.
One diploid cell showed no karyotypic abnormalities.
Eleven months after the first resection a biopsy specimen of a recurrent tumor was obtained which showed the same modal karyotype.
The modal karyotype of both resections was: 44,X, -Y, +1p-, -9, 11p+, -14.

Case 7.: Mode 47. Range 44-86.
Three G-banded and two C-banded metaphases were analyzed. In all cells examined trisomy # 20 was present.
Six months after the first resection, a biopsy specimen of a recurrent tumor at the primary site was obtained which showed the same karyotype.
There were a few near tetraploid cells, which were unsuitable for chromosomal analysis.
The modal karyotype of both resections was: 47,XY, + 20.

Case 8.: Mode 42. Range 41-86.
Ten G-banded and two C-banded metaphases were analyzed. Missing chromosomes were one copy of # 3, # 6, # 7, # 17, one X-chromosome and both copies of # 9. A dicentric translocation, tdic (7;9)(p11;p11)[7qter→p11::9p11→qter] and two marker chromosomes were present. Mar 1, a large marker, was composed of most of a chromosome # 3 and the short arm (including the centromere) of # 6. Between these parts a homogeneously staining region (HSR) was present. With C-banding the presence of the two centromeres was demonstrated. The composition of this marker is probably: 3qter→p13;HSR;6q13→pter. Mar 2 is a derivative 9 chromosome and is probably a t(9;17)(q34;q21?) [9pter→ q34::17q21? → qter?].
A cell with 57 chromosomes was also fully karyotyped. The HSR containing marker and two of the dicentric translocation chromosomes as described were present. Besides, a small metacentric marker chromosome was seen.
In the metaphases with about 85 chromosomes, present in 8 out of 60 cells, two HSR containing markers were present. Further karyotyping was impossible.
The modal karyotype was: 42,X, -X, -3, -6, -7, -9, -9, + t(7;9), -17, + mar 1, + mar 2.

Case 9.: Mode 60. Range 52-63.
Six G-banded and two C-banded metaphases were analyzed. Consistent features were: a translocation t(1;6)(p12;p12) [6pter---›p12::1p12 ---›1qter], del 2(q12), 4p+, del 9(q13), 11p+, 12p+, two abnormal derivative 13 and 14 chromosomes, 17q+, 19q+, 20q+ and three different markers. Chromosomes # 1, # 3, # 7, and # 17 were present in triplicate. Chromosomes # 2, # 10, # 12, # 14, were present in single copies. No normal # 13 and # 14 were seen.
The modal karyotype was most probably: 60,XY,+X, + 1, + t(1;6) (p12;p12), 2q-, +3, +4q+, +7, +9q-, -10, +11p+, 12p+, 13p+, 13p+, 14p+, 14p+, +17, +17q+, +19q+, +20q+, + mar 1, + mar 2, + mar 3.

Case 10.: Mode 83. Range 64-85.
Four G-banded and two C-banded metaphases were analyzed. Consistent features were 5 structurally abnormal chromosomes, three of which could be identified to some extent: 12q+, 6q-, 21p+?. The remaining two were qualified as markers.
Therefore, the modal karyotype was most probably: 83,XX,+X, +1, +2, +3, +3, +4, +5, +6, +6q-, +7, +8, +8, +9, +10, +10, +11, + 11, +12, +12q+, +13, +14, +15, +16, +17, +17, +18, +19, +19, +20, +20, +21, +21, +21p+?, +22, +22, + mar 1, + mar 2.

Case 11.: Mode 77. Range 46-87.
Four G-banded and two C-banded metaphases were analyzed. Consistent features were: an extra 1q- [del (1)(q12?)], a large 11p+ chromosome composed of most of chromosome # 11 and the long arm of # 1, including the centromere. Between these two chromosome parts, a HSR was present. The probable composition of this marker was: 11qter→p14?;HSR;1p12?→ 1qter.
Eleven markers varying in length from the A- to G-group were present.
Most chromosomes were present in triplicate, except for # 2, # 4, # 8, # 10, which were normal, and # 22 showing four copies.
The modal karyotype was most probably: 77,XX, +1, +1q-, +3, +5?, +6, +7, +9, +11p+, +12, +13, +14, +15, +16, +17, +18, +19, +20, +21, +22, + 22, + mar 1, + mar 2, + mar 3, + mar 4, + mar 5, + mar 6, + mar 7, + mar 8, + mar 9, + mar 10 + mar 11.

Case 12.: Mode 71. Range 53-76.
Two metaphases were completely and three partially karyotyped after G-banding. Two C-banded cells were available. Striking features were: 1q-(q22), 2q-(q14), 5p+, 8p+, 10p+, 12p+.
Fourteen marker chromosomes, varying in length from the A- to G-group were present.
The modal karyotype was most probably: 71,XY, +Xq+, +1, +1q-, +2q-, +3, -4, 5p+, +7, 8p+, +10p+, +11, 12p+, +13?, +17, +17, +21, + mar 1, + mar 2, + mar 3, + mar 4, + mar 5, + mar 6, + mar 7, + mar 8, + mar 9, + mar 10, + mar 11, + mar 12, + mar 13 + mar 14.

Case 13.: Mode 73. Range 63-79.
Five G-banded and two C-banded metaphases were analyzed. Consistent features were two, and in some cells three, 9q+ chromosomes. It appeared that these derivative chromosomes showed two centromeres, one of which was suppressed [psu dic (9)]. An isochromosome i(2q) and two markers were present.
The modal karyotype was most probably: 73,X,+X,-Y, +1, +1, -2, +i(2q), +6, +6q-, +7, +8, +8, +psu dic (9), +psu dic (9), +10, +11, +11, +12, +12, +13, +13, +14, +14, +15, + 15, +17, +18, +18, +19, +20, +mar 1, +mar 2.

Discussion

As reported in a previous study, non-infiltrating bladder tumors mostly have a modal chromosome number and a chromosomal range in the near diploid region, whereas infiltrating cancers mostly show hyperdiploidy [9].

In this study representative karyotypes of the modal chromosome numbers or 13 G-banded tumors are given.

In three (cases 5, 6 and 13) of the ten males the Y chromosome was missing. In bone marrow this is frequently observed and could be an age dependent phenomenon [10]. The ages of our three patients where 54, 73 and 90 years, respectively. In three of four primary gastric cancers, recently published, the Y chromosome was missing. The age of these patients was 27, 68, 54 years, respectively, whereas in the fourth patient, age 74 the Y

chromosome was present [11]. Our data suggest that the loss of the Y chromosome is not merely related to age.
The autosomes most frequently involved in structural and/or numerical aberrations are in numerical sequence:
Chromosome # 1: This chromosome was involved in structural rearrangements in 6 cases (Table 2) (Fig. 1). Also, in published series of bladder cancer, chromosome # 1 frequently underwent structural changes [2, 3, 12-14]. Furthermore, abnormalities of # 1 are reported as occurring in various other types of human neoplasia [15-19]. However, there is much variation in the position of the breakpoints. The eleven recognized breakpoints in the cases of bladder cancer, cited above and in our cases, are between 1p22 and 1q25, i.e. in the proximal regions of the chromosome arms, consisting of approximately of half of the entire chromosome.

Chromosome # 2 showed structural changes in four cases: in three a deletion of part of the q-arm and in one an i(2q) were observed. The involvement of chromosome # 2 is not striking in the reports of previous authors dealing with bladder tumors. On the other hand in malignant melanoma structural aberrations of # 2 are frequent [19].

Chromosome # 3 was involved in a structural change in one case and in four other cases it was present in triplicate.

Chromosome # 6 was frequently involved. In case 9 a translocation, t(1;6) (p12;p12), and in case 8 a translocation t(3;?;6)(p13;HSR;q13), were seen. Trisomy 6 was present in four near triploid cases. In the study of Gibas et al. [3] chromosome # 6 was affected in five of the nine tumors, mostly as a deletion of the long arm. Deletions of the long arm of chromosome # 6 are often found in malignant disorders [20]. Involvement of chromosome # 6 in structural or numerical alterations of the karyotype, was accompanied by other chromosomal changes in all cases including ours. Thus we agree with other authors [3, 21]. that abnormalities of chromosome # 6 most likely are secondary changes.

Chromosome # 7 was involved in structural rearrangements in one case and in the five near triploid tumors as an additional chromosome. Trisomy # 7 has been reported as a possible primary change in TCC of the ureter [4]. In bladder cancer this change was present in two near triploid cases of Atkin and Baker [2] and in one near diploid and one near triploid case of Gibas et al. [1, 3]. Because trisomy # 7 was absent in our near diploid cases and absent in all (except one) near diploid bladder tumors of the studies cited above, we tend to consider trisomy # 7 as a secondary change.

Chromosome # 8 was involved in structural changes in two cases. An isochromosome i(8q), as in our case 4, has been reported before by Gibas et al. [1, 3] in two cases.

Chromosome # 9 was involved in five of the eight near diploid cases: (9q-; -9; 9q+; -9; -9, -9, +t(7;9) respectively). Atkin and Baker [2] found a missing # 9 in three of six near diploid bladder tumors and in one of four strongly hyperdiploid ones.
Gibas et al. [1] reported monosomy # 9 in three of six near diploid tumors and in one of three near tetraploid cases. In the following study of this authors [3] monosomy # 9 has been reported in one of four near diploid cases.
Berger et al. [4] found a missing # 9 in two of six near diploid cases. The case described by Kovacs [13] and the diploid case examined by Vanni et al. [22] also showed a missing # 9.
Taken together, the cited studies and our data, in 14 of 32 near diploid bladder tumors

chromosome # 9 was missing and in two other cases a structural aberration was recognized (Table 3). It is more and more likely that loss of chromosome # 9 is a primary change in bladder tumor.

In two cases a deletion of the long arm of # 10, breakpoint at q22, was present. This is very interesting since Gibas et al. [1] and Berger et al. [4] each reported a patient with a deletion of 10q(24). The deletion in one of our cases was the sole abnormality, in the other it was accompanied by monosomy # 9.
Deletion of # 10 could be a primary change, since it was found in two near diploid tumors.

Chromosome # 11 was involved at least three times in structural rearrangements; in one case with a near diploid chromosome count and in two near triploid cases. Thus, it appears to us, that chromosome # 11 is preferentially associated with invasiveness and that it is not involved in the initial steps of carcinogenesis of the bladder. Atkin and Baker [2] reported frequent structural changes of # 11, including short arm deletions. This could be of interest since the oncogene c-Ha-ras$\hat{1}$ is located on 11p14 [23]. In our cases we were unable to recognize the breakpoint in # 11 rearrangements.
Deletions of 11p are well known to occur in tumor cells of patients with Wilms' tumor [24].
Two of our cases (cases 8 and 11) contained homogeneously staining regions (HSR). This phenomenon has been described in a variety of tumors and is related to gene amplification [25, 26]. It may be a reaction of exposure to drugs [27]. However, our cases were primary tumors and the patients did not receive chemotherapy before resection of the tumors mentioned.
Atkin and Baker [2], using a direct technique, revealed involvement of chromosome # 11 as structural change in all six near diploid cases. In the eight cases examined by Berger et al. [4], data obtained after enzymatical disaggregation of the specimens and culturing the malignant cells, no involvement of # 11 was seen. Our results show an abnormal chromosome # 11 in one of the near diploid tumors.
The simultaneous application of both methods, to more tumors is necessary to draw conclusions about the significance of chromosome # 11 in bladder carcinogenesis.

Chromosome # 17 had undergone structural aberrations in two cases and was present in three or more copies in all near triploid cases. The involvement of this chromosome has been reported before [2].

In one case an additional chromosome # 20 was seen as a sole abnormality. In a resection specimen obtained six months after the first, the same karyotype was present. Atkin and Baker [2] reported on the presence of one or more extra # 20, together with other chromosomal changes. Berger et al. [4] reported this in two bladder tumors, but an extra # 20 was even more frequently found in renal tumors. They interpreted trisomy # 20 as a possible secondary change.
Our findings confirm those of others in some cases, but it is also clear that differences were observed. The most obvious example is the absence of i(5p). This isochromosome was reported by Gibas et al. [1, 3] in 7 out of 16 tumors. It cannot be established with certainty that i(5p) was missing in our study. For instance, in case 8 a small metacentric chromosome was present which resembles i(5p) or a deletion of 5q at q13. However, both copies of chromosome # 5 present were normal.

In some cases normal diploid cells were present. Presumably, they represent normal stromal, epithelial or muscle cells.

The number of translocations found in this study was low. Furthermore, different breakpoints in different chromosomes were involved. Therefore, specific translocations were not observed.
Reciprocal translocations characterise several forms of leukemia and lymphoma and contribute to the process of neoplastic transformation by activating oncogenes situated close to the breakpoints [21, 28, 29]. Solid tumors in contrast, generally present unbalanced chromosomal changes, rather than reciprocal translocations. This results in duplication or loss of (parts of) chromosomes [30]. This mechanism is relevant for the acquisition of cancer genes in hemizygous form. As suggested by Knudson [31] invisible mutations - which can lead to homozygoty for recessive cancer genes - or loss of genetic material by deletion of (a part of) the chromosomes - which leads to hemizygoty of the recessive cancer gene - can produce cancer, as demonstrated in Wilms' tumor and retinoblastoma. Further support for Knudson's model has been obtained at the DNA level [32].

To find chromosomal changes which are specific or directly related to the origin of the tumors, near diploid tumors are needed, with preferably a few or ideally one chromosome abnormality. In spite of the problem of many chromosome abnormalities in poorly differentiated tumors, it is worthwhile to examine these tumors as well, since possible specific rearrangements could be found, which are indicators for aggressiveness of the tumor [28].
To establish the primary chromosome change, direct cytogenetic examination offers the best chance of success, in spite of the fact that after culturing the cells, banding of the chromosomes is better. Chromosomal changes in vitro and growth advantage of a special cell line include the risk of contradictory data.
This study stresses loss of chromosome # 9 as a probable primary change. Deletion of chromosome 10q at band q22 is possibly another primary change.
If more well differentiated transitional cell carcinomas of the bladder are chromosomally analyzed, it might be possible to divide bladder cancer in subgroups analogous to the situations in the acute leukemias.
Prospectives in the field of therapeutic approaches and clinical behavior of the tumors also may be improved.

Table 1.

Summary of the clinical data, range and modal chromosome number.

case	age	sex	stage/grade	chromosomes range	chromosomes m.n.
1.	74	M	TaG2b	43-90	46
2.	64	M	TaG2a	46	46
3.	80	M	TaG2a	44-45	45
4.	33	M	TaG2b	47	47
5.	73	M	Ta(m)G2a	42-46	46
6A.	54	M	T1(m)G2a	41-72	44
B.	55	M	TaG2b	41-75	44
7A.	74	M	T1G2b	44-86	47
B.	74	M	T1G2b	45-83	47
8.	87	F	T2G2b	41-86	42
9.	74	M	T2G3	52-63	60
10.	79	F	T2G2b	64-120	83
11.	67	F	T3G3	46-87	77
12.	69	M	T3G3	53-76	71
13.	90	M	T3G3	63-79	73

m.n. = modal number of chromosomes.
(m) = multiple tumors

Table 2.

Summary of the most frequently involved chromosomes and their abnormalities.

case	m.n.	1	3	7	9	10	11	17
1.	46					10q−		
2.	46				9q−			
3.	45				−9	10q−		
4.	47				9q+			
5.	46	+1p−						
6.	44	+1p−			−9		11p+	
7.	47							
8.	42		−3	−7	−9			
			t(3;II;6)	t(7;9)	−9			
					t(9;17)			−17
9.	60	+t(1;6)	+3	+7	+9q−	−10	+11p+	+17
		+						+17q+
10.	83	+1	+3	+7	+9	+10	+11	+17
			+3			+10	+11	+17
11.	77	+1q−	+3	+7	+9		+t(11;H;1)	+17
		+1						
12.	71	+1q−	+3	+7		+10p+	+11	+17
		+1						+17
13.	73	+1		+7	+9q+	+10	+11	+17
		+1			+9q+		+11	

Table 3.

Summary of the near diploid tumors and the most frequently involved chromosomes.

A. Culture method:

author(s)	all tum.	dipl. tum.	case	m.n.	chromosome # involved						
					1	5	6	8	9	10	11
Gibas '84	9	6	1	48		+i(5p)		i(8q)			
			2	45	p+		q–		–9	p+	–11
			3	44				p–		q+	
			5	47					–9		
			6	47		+i(5p)	q–			q–	
			7	45					–9		
Gibas '86	7	4	1	45	q+				–9		
			2	47							
			3	43	p+;q–	i(5p)	q–				p–;p+
			5	48		q–	t(6;X)				t(11;13)
											q–
Berger '86	8	6	1	46				p–	–9;i(9q)		
			2	45							
			3	46							
			4	46						q–	
			6	46					–9		
			7	47							

B. Direct method:

author(s)	all tum.	dipl. tum.	case	m.n.	chromosome # involved						
					1	5	6	8	9	10	11
Vanni '85	2	1	1	49	q–				–9		p+
Kovacs '85	1	1	1	46	+p–				–9		
Atkin '85	10	6	1	42/3							i(11q)
			2	42/5	+p–		+6		–9		t(11;14)
			3	44/5	q+		q–				–p
			4	46				–8	–9	–10	q+
			5	46	+i(1q)			p–	–9	–10	p–
			6	46	q+						r(11)
This study	13	8	1	46						q–	
			2	46					q–		
			3	45					–9	q–	
			4	47				i(8q)	q+		
			5	46	+p–						
			6	44	+p–				–9		p+
			7	47							
			8	42			–6 t(3;H;6)		–9;-9; t(7;9) t(9;17)		

All tum. : patients examined; near diploid and strongly hyperdiploid tumors.
dipl. tum.: number of tumors with a near diploid chromosome number.
m.n. : modal number.

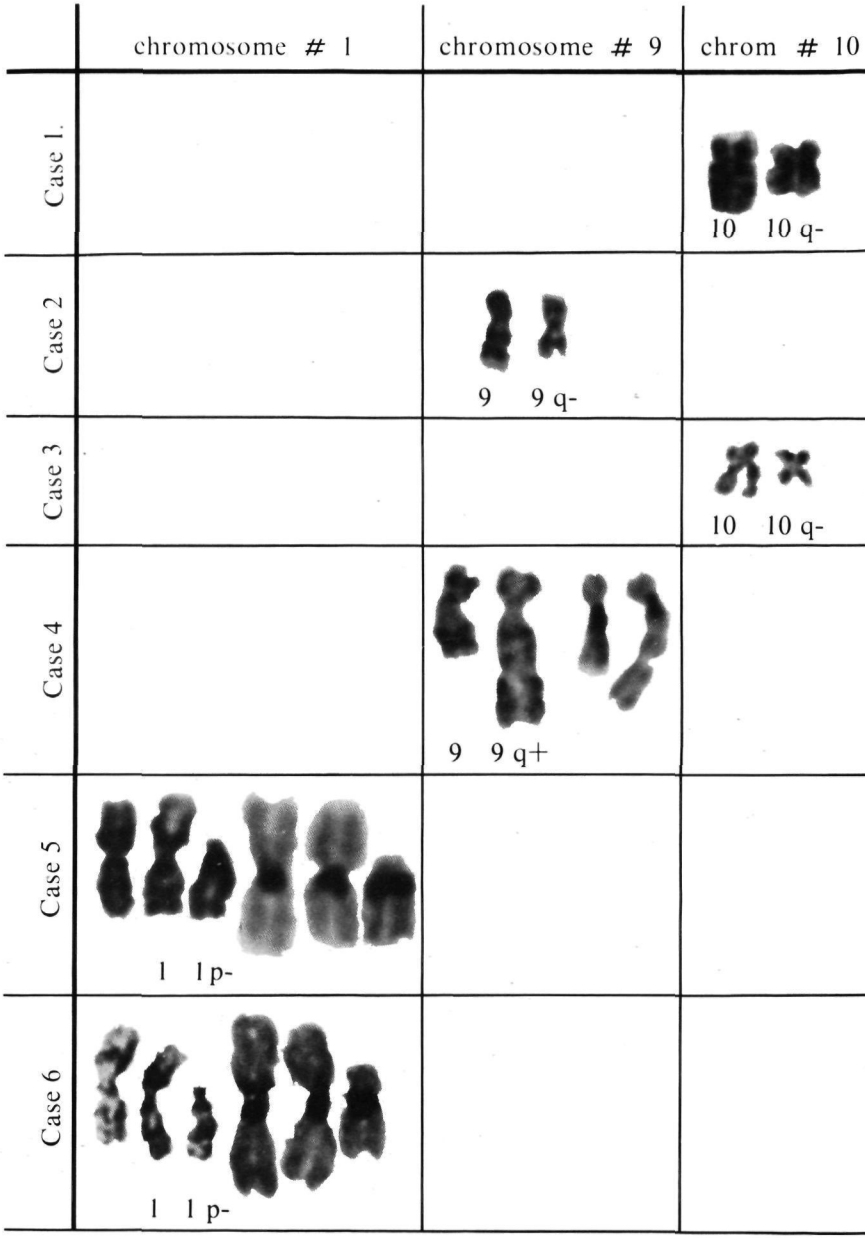
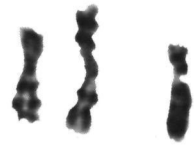
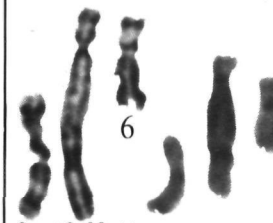
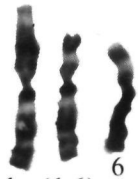
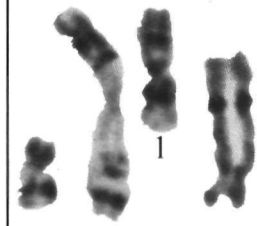
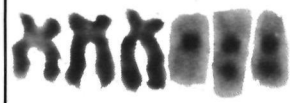


Figure 1
Structural abnormal G-banded chromosomes derived from # 1, # 9, # 10 and two HSR containing chromosomes. In a number of cases the C-banded chromosomes are placed on the right side.

	chromosome # 1	chromosome # 9	HSR
Case 8		 7 t (7;9) t (9;17)	 6 3 t (3;H;6)
Case 9	 6 1 t (1;6)	 9 9 q-	
Case 11	 1 1 q-		 1 11 t (11;H;1)
Case 12	 1 1 q-		
Case 13		 9 9q+ 9q+	

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Chapter VI

Blood group isoantigen deletion and chromosomal abnormalities in bladder cancer

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Submitted.

Abstract

Presence or absence of blood group isoantigens (BGI) in 78 cases of transitional cell carcinoma of the bladder was correlated with tumor stage and grade and results of chromosomal analysis. For BGI detection the indirect immunoperoxidase method with monoclonal antibodies to A, B and H antigen was used. In 51 percent of the 59 superficial tumors blood group isoantigens were demonstrable, whereas all deeper infiltrating and higher graded tumors were BGI negative. However in superficial tumors the mode of BGI expression did not correlate significantly with tumor recurrence and progression. Although a consistent correlation was demonstrated between chromosomal numbers and clinical course, the chromosomal abnormalities found and mode of BGI expression, even in combination, had no prognostic value additional to the grading criteria used. Of 7 superficial tumors the karyotype could be analyzed.

Introduction

Superficial transitional cell carcinomas (TCC) of the bladder with identical histopathology can vary significantly in their subsequent clinical course. [1] A significant degree of inter- and intra- observer variability especially in the evaluation of tumor grade is reported. [2] Blood group isoantigen (BGI) deletion and chromosomal abnormalities are mentioned as prognostic parameters additional to tumor stage and grade. [3,4] With respect to BGI content and tumor behavior in bladder cancer contradictory results have been published. According to some authors, loss of these antigens occurs especially in tumors which carry a poor prognosis with respect to invasion and metastasis. [4,5-7] Other authors observed that a significant number of invasive urothelial cell carcinomas was strongly positive for A, B and H antigens. [8,9] These differences might be accounted for, at least in part, by the method used. [7,10] The initial studies on BGI-deletion employed the specific red cell adherence (SRCA) test. [5] Later, the indirect immunoperoxidase method was introduced. [7] The reproducibility of this technique has been limited by the need to use a lectin in detecting the H isoantigen, together with variation in specificity found with conventional polyclonal blood group antisera.
The availability of monoclonal antibodies (McAb's) has led to the production of highly specific reagents especially superior in the demonstration of the H isoantigen. In previous studies a correlation between increased chromosomal numbers and tumor invasion and grade [11,12] was shown (this thesis chapter II, III and IV). In the present study the presence or absence of blood group antigens is correlated with tumor grade and stage and chromosomal abnormalities. In addition follow up in patients with a superficial bladder tumor in relation to BGI expression is studied.

Materials and methods

The initial biopsies from 78 patients presenting with a primary urothelial cell carcinoma of the bladder between 1980 and 1987 were studied (see table 1). Staging was done according to the T.N.M.-system.[13] Tumors were called grade 1 if they showed increased cellularity with or without slight cellular and nuclear variations. Grade 2 tumors according to the criteria used showed increased cellularity, clear cytologic deviation and a tendency to loose

Table 1
Distribution of stage and grade among analyzed tumors

Tumor grade	Tumor Stage			Total
	Ta	T1	T2-4	
Grade 1	29	1	-	30
Grade 2	9	15	8	32
Grade 3	-	5	11	16
Total	**38**	**21**	**19**	**78**

the normal polarity. Grade 3 applied to tumors with the most severe degree of cellular dysplasia.

When progression in tumor stage was seen, or treatment was done with chemotherapy, immunotherapy or radical therapy, patients were excluded from further follow up.

Determination of blood group isoantigens

Determination of ABH blood group isoantigens was done with the indirect immunoperoxidase method [14] with the use of monoclonal mouse anti-A (Dako A 571), anti-B (Dako A 581) and anti-H (Dako A 582) antibodies.

Paraffin sections cut a 5 μm, were deparaffinized and, after blocking endogeneous peroxidase activity with 0.3% H_2O_2 in absolute methanol, rehydrated and washed in phosphate-buffered saline solution (PBS). The sections were then incubated for 15 minutes with normal rabbit serum (NRS) 20% in PBS to block non-specific antigen sites. After rinsing in PBS, sections were incubated overnight at 4° C with the appropriate McAb, diluted 1 100 in 1% bovin serum albumin (BSA) in PBS, pH 7.2 After washing in PBS twice for 15 minutes each time, the sections were incubated with rabbit anti mouse immunoglobulin horse-radish peroxidase (HRP) conjugated (Dako P 260) 1:20 in 1% BSA in PBS The tissue sections were then washed twice in PBS followed by incubation in diaminobenzidine, DAB (0 05% DAB + 0.01% H_2O_2 in PBS pH 7 6), washed and counterstained with Haematoxyline Mayer. After washing in tap water the sections were dried and mounted with Permount.

Tumors were considered positive for B.G.I. when by microscopic examination distinct tumor cell membrane staining could be discerned in at least 5% of the cancer cells in a slide. Positive and negative controls were used as described by others.[14] In mucosal biopsies from 3 patients none or only very faint staining of normal urothelium in non tumor bearing areas was seen. These patients were not included in this analysis as they were probably nonsecretors. Examination of the slides was done independently and since 1983 prospectively by two pathologists, unaware of the results of chromosomal analysis and follow up of the tumors.

Chromosomal analysis

For chromosomal analysis the direct method and, in most cases, short term culture as well, was used.[15] Staining was done with Giemsa. If sufficient good metaphases were available, banding methods were used. Analyzable metaphases were karyotyped according to the Denver nomenclature.[16]

Unless otherwise mentioned, statistical analyses were done with the chi square test.

Results

Blood group antigen - antibody reaction

The use of the immunoperoxidase method with the appropriate McAb's resulted in all analyzed cases in the formation of a distinct brownish red staining of the cell membranes of normal urothelium, vascular endothelium and erythrocytes, used as internal controls (see fig. 1). Background staining was very low. In tumor tissue, the pattern of blood group antigen-antibody staining appeared variable. In superficial tumors an antigen-antibody reaction varied from the typical staining pattern in all malignant cells to staining of only a few cells of the basal or, in others, the luminal layer (see fig. 2). In addition, the intensity of staining was not uniform. In tumors classified as BGI- positive, the surface area with membrane staining ranged from 5 to 90 per cent (see table 2). The difference in mode of BGI expression regarding tumor surface area between non-invasive and invasive as well as between low grade and high graded tumors, as shown in table 3, is highly significant ($p = 0.001$).

Table 2
Distribution of estimated tumor surface area with BGI-expression according to tumor stage and grade

Tumor surface area percentage positive	Tumor stage and grade					
	TaG1	TaG2	T1G1	T1G2	T1G3	T2-4
0	9	6	-	9	5	19
5-9	1	1	1	2	-	-
10-19	8	1	-	1	-	-
20-29	2	-	-	2	-	-
30-39	5	1	-	1	-	-
40-49	2	-	-	-	-	-
50-90	2	-	-	-	-	-
Total	**29**	**9**	**1**	**15**	**5**	**19**

Table 3
Correlation between BGI-expression and tumor stage and grade

BGI expression	Tumor stage			Tumor grade		
	Ta	T1	T2-4	G1	G2	G3
Positive	23	7	-	21	9	-
Negative	15	14	19	9	23	16

Cytogenetic Analysis

With respect to the chromosome number (mode and range) we found a significant difference between non-infiltrating and low grade tumors on the one side, and infiltrating or higher graded tumors on the other side ($p < 0.001$; table 4). In infiltrating, grade 2 and grade 3 tumors nearly always cells with more than 49 chromosomes were found, even if the modal number was (near) diploid ($p < 0.01$). Although marker chromosomes, found by routine chromosomal analysis, were significantly more frequently seen in tumors of

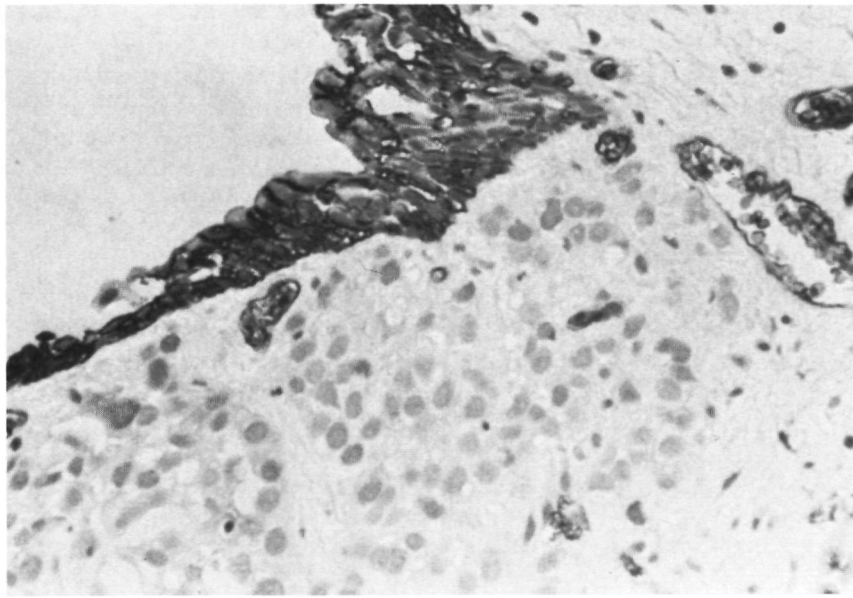

Fig. 1 TCC stage T1 grade 2. Normal urothelium, vascular endothelium and erythrocytes positive immunoperoxidase staining for blood group substance; tumor neg. (x 250, counterstaining with haematoxyline Mayer).

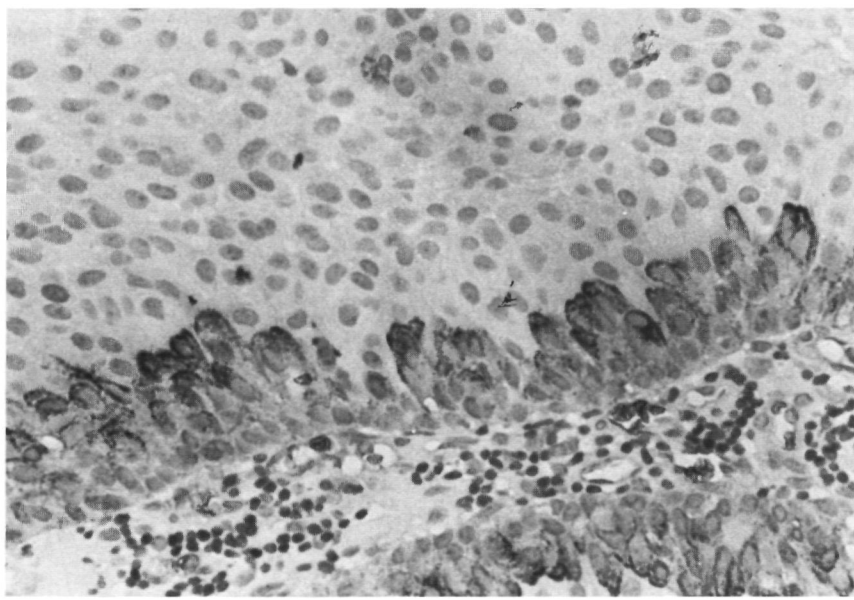

Fig. 2 TCC stage Ta Grade 1 showing immunoperoxidase staining for blood group substance; only cells of the basal layer are positive (x 250, counterstaining with haematoxyline Mayer).

higher stage ($p = 0.06$) or grade ($p = 0.0001$), they were also often found in low stage, low grade tumors (see table 4).
With direct method and G- and C-banding methods, the karyotype of 7 superficial tumors could be analyzed (see table 5). In 4 of these tumors chromosome number 9 was involved. In two cases a deletion of the long arm of chromosome number 10 was found, in two other male cases chromosome Y was missing.

Relation of BGI-expression with Chromosomal Analysis

No significant correlation between BGI-expression and modal number ($p = 0.59$), chromosomal range ($p = 0.13$) or presence or absence or marker chromosomes ($p = 0.26$) was demonstrable in superficial tumors.

Correlation of tumor markers with clinical course

Thirty three patients with a non-infiltrating tumor were evaluable for analysis with respect to clinical course (see table 6). Follow up was 12 to 66 - mean 34 - months. The percentage of patients free of recurrences or tumor progression was consistently lower among patients with a grade 1 tumor, and among tumors with (near) normal chromosomal numbers. No significant difference was demonstrable in clinical behavior whether or not blood group antigens were present in the initial tumor. Tumor progression was seen in 2 patients in which 30 per cent of the tumor surface area was clearly BGI positive.
Most patients with a T1 grade 2 lesion were, after initial tumor resection or after their first recurrence, treated with intravesical chemotherapy or immunotherapy and thus not available for further follow up. Of the remaining 7 patients, progression was seen in 4 within 4 to 8 months. In 2 of these patients the initial tumor was BGI positive.
The correlation of tumor grade and chromosomal numbers in combination with BGI-expression with respect to progression in non-infiltrating cancers is shown in table 7.
Statistical analysis (within a logistic linear regression model) of the figures presented in this study shows that, with respect to the occurrence of recurrences or tumor progression, the number of chromosomes, presence or absence of marker chromosomes, or the mode of BGI-expression had no prognostic value additional to tumor grade.
A summary of the detailed histopathologic and cytogenetic data for individual bladder tumor patients is as an addendum added to this report.

Table 4
Tumor stage and grade in relation to cytogenetic data

Tumor stage and grade	Chromosome number			
	mode		range	
	≤49	>49	≤49	>49
Ta	33(12)*	5(4)	29(8)	9(8)
T1	9(6)	12(8)	-	21(14)
T2-4	6(1)	13(8)	-	19(9)
Grade 1	29(8)	1(0)	26(5)	4(3)
Grade 2	17(10)	15(13)	3(3)	29(20)
Grade 3	2(0)	14(8)	-	16(8)

( )* number of tumors with marker chromosomes.

Table 5
Mode of BGI expression and karyotype in 7 superficial tumors

Case	Sex	T-G	BGI Expression	Karyotype
1	M	TaG1	neg.	46, XY, 9q-, -12, + mar.
2	M	TaG1	10% pos.	45, XY, -9, del(10)(q22)
3	M	Ta(m)G1	neg.	46, X, -Y, +1p-
4	M	TaG2	neg.	47, XY, 2q-, +i(8q), 9q+
5	M	T1(m)G1	neg.	44, X, -Y, +1p-, -9, 11p+, -14
6	M	T1G2	neg.	46, XY, del(10)(q22)
7	M	T1G2	neg.	47, XY, +20.

Table 6
Correlation of tumor markers with follow up* for Ta tumors

Number of	Grade		Chromosomal Mode		Chromosomal Range		Chromosomal Marker		BGI Expression	
patients	1	2	≤49	>49	≤49	>49	−	+	+	−
Evaluable	27	6	30	3	26	7	21	12	21	12
Disease free(%)	15(56)	1(17)	16(53)	0	14(54)	2(29)	12(57)	4(33)	12(57)	4(33)
Non-invasive recurrence(%)	10(37)	3(50)	11(37)	2(67)	10(38)	3(42)	8(38)	5(42)	7(33)	6(50)
Tumor progression(%)	2(7)	2(33)	3(10)	1(33)	2(8)	2(29)	1(5)	3(25)	2(9)	2(17)
Recurrence rate	2.5	4.2	2.8	2.0	2.8	2.8	2.0	4.0	2.4	3.4

* Follow up varying from 12 to 66 - mean 34 - months.

Table 7
Correlation of tumor markers in combination, with progression, in 35 patients with TCC stage Ta

	No of patients							
	Chromosomal						BGI expression	
	mode		range		markers		positive*	negative**
	≤49	>49	≤49	>49	no	yes		
Grade 1	26/2[1]	1/0	24/1	3/1	21/1	6/1	19/2	8/0
Grade 2	4/1	2/1	2/1	4/1	0/0	6/2	2/0	4/2
BGI pos.*	21/2	0/0	18/1	3/1	14/1	7/1		
BGI neg.**	9/1	3/1	8/1	4/1	7/0	5/2		

[1] Values are given as number of patients evaluable/numbers of patients with tumor progression.
Follow up 12 to 66 - mean 34 - months.

Discussion

Several technical problems regarding detectability of blood group antigens and reproducibility of test results made widespread clinical application of BGI-analysis

inappropriate. It was hoped that the use of monoclonal antibodies might solve some of the problems.[9,17] In our study false negatives were ruled out by the presence of strongly positive internal controls. Patients in whom no staining of normal urothelium was seen, were excluded from this study. Probably these patients were nonsecretors. The expression of blood group specificities in normal (secretory) epithelium and in epithelial tumors is strongly related to the secretor status of the person.[18,19] In urothelium, the A, B and H antigens are present in much lower amounts or even absent in the 15-20 per cent nonsecretor persons. That means a restriction in the clinical usefulness of ABH antigenicity in bladder tumors.

A major problem is how the test results should be quantified. Interpretation of the BGI test is subjective and is not as straight forward as indicated in many reports. Loss of blood group antigens is not an "all or none" phenomenon.[20] Often an incomplete reaction is seen, indicating presence of antigens in some areas and absence in other areas in the same block of paraffin embedded tissue or even in the same field. The detectability of BGI can be influenced not only by the method of tissue processing and the type of anti-sera, but also by inherent differences in the quantity of BGI on the tumor cell surface.[8,20,21] The action of malignant cells might be to suppress to a variable extent the conversion of H to A and B antigens and perhaps the formation of H from precursor substance.[8] The intratumor heterogeneity of blood group antigen expression complicates the interpretation of test results regarding BGI deletion which is another restriction for the clinical usefulness of ABH-antigen measurements. Because of the inherent variability in staining reaction, it is not surprising that various authors have differed in their definition of a positive test result (i.e. BGI retention). Since the results of both the specific red cell adherence (SRCA) test and immunoperoxidase methods are based on semiquantitative evaluations of light microscope preparations, the cutoff point for non-reactivity can not be objective. Weinstein and associates [22] stated that tumors with less than approximately 30 per cent of the cells expressing the ABH antigens as shown with polyclonal antibodies have the same high probability to invade the bladder wall as those with no antigen staining. Giraldo and associates [9] established the 30 per cent criterion after determining that the degree of background staining never exceeded 30 per cent. In a study on BGI deletion with monoclonal antibodies in cervical carcinoma it was shown that cases with positive staining in between one and 30 per cent of cells, showed the same survival as the more positive cases.[23] In our material with a threshold for positivity as low as 5 per cent all BGI positive tumors were non- or only superficially invasive, whereas all deeper infiltrating and all grade 3 carcinomas were negative for ABH antigen. So a good correlation between test results and tumor stage and grade was demonstrable.

With respect to tumor recurrences and progression in superficial tumors, we could not find a consistent difference between BGI positive and BGI negative tumors, irrespective of the threshold for positivity. Tumor progression was seen in BGI positive as well as in BGI negative tumors. Other authors also concluded that evaluation of blood group antigens could not be equated to biological behavior in transitional cell carcinomas.[8,9,24-26] The lack of an absolute correlation in their studies, like in our own, is rather significant, thus eliminating the use of this technique in treatment planning for the individual patient. It has been proposed that the loss of blood group antigens in cystoscopically normal epithelium may represent one of the earliest measurable changes of the malignant potential of the urothelium.[27,28] ABH antigenicity of normal looking epithelium was

mentioned to be a better predictor of recurrences the first year after initial diagnosis than other prognostic indices including tumor grade and histological status of the mucosal biopsies.[28] The predictive value of ABH antigenicity of mucosal biopsies with respect to tumor progression is still unknown.

When more is known about the genetics and biochemistry of ABH antigens and their precursors in normal and neoplastic epithelial cells, perhaps a precursor substance is found, common to all individuals, irrespective of the ABO and secretor types. Determination of presence or absence of a common precursor might increase the prognostic accuracy of blood group antigen analysis in bladder cancer.[29]

Immunohistochemical studies have also explored the relation between the expression of other blood group antigens and the biological behavior of bladder cancer. With respect to the Thomsen Friedenreich antigen, conflicting results have been reported.[4 30 31] The prognostic significance of Lewis a and Lewis b antigens in superficial bladder tumors still has to be defined.[32]

Cytogenetic analysis of bladder cancer showed that low grade and noninvasive tumors generally have hypo- or peridiploid chromosomal numbers.[4,12] In high grade tumors hyperdiploid modal numbers are found. Intermediate grade tumors often are characterized by (near) modal numbers, but as we showed before,[12] in these tumors nearly always hyperdiploid cells are demonstrable. Thus the range in chromosomal numbers appeared to be an objective basis for grading. From the present study it appeared that the finding of marker chromosomes, unidentified structurally abnormal chromosomes, has no prognostic value additional to tumor grade or chromosomal range. In our view the finding of marker chromosomes is to a high degree dependent on technical methods. Identification of chromosomes by banding methods is needed to evaluate whether specific chromosomal abnormalities are related to future malignant behavior of the primary tumor analyzed.

A detailed study about our results with chromosome banding methods in infiltrating and non-infiltrating bladder cancers is reported elsewhere.[33] In 4 of the 7 superficial tumors in which we were able to analyse the karyotype, chromosome number 9 was involved. The genes for the ABO blood groups are located on chromosome 9.[34] Loss of chromosome 9 is often reported in bladder tumors [35-38] and might be a primary change in urothelial cell carcinoma.[32] We found various karyotypes at first diagnosis by direct method, probably indicating the existence of subtypes of bladder tumors. Follow up of these karyotyped tumors is as yet to short to evaluate a prognostic meaning.

Chromosome analysis and mode of BGI expression are claimed to be the most reliable tumor markers for bladder cancer.[4,39] From the present study it is clear that, with respect to future tumor behavior, the chromosome number, chromosomal markers, and the presence or absence of ABH blood group expression have no prognostic value additional to tumor grade with the grading criteria used. So far tumor stage and grade remain the hallmarks in assessing the malignant potential of superficial transitional cell carcinoma of the bladder.

Dr W Doesburg and A. Reintjes, University of Nijmegen, performed statistical evaluation, miss L Laarakkers supplied technical assistance; Drs J Scheepers and R Retera and the members of the Operating Room provided cooperation

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Histopathologic and cytogenetic data of individual bladder tumor patients

Patient				Tumor			Chromosomal							Follow up	
Number	Age	Sex	Blgr [1]	T	G	B.G.I. Pos. [2]	Mode	Range	Ploidy [3] n	2n	3n	4n	5n	Marker	Rec./ Months [4]	Progression [5]
1	51	M	B	a(m)	1	–	43	40-45		40				+	3/41	–
2	27	M	A	a	1	10%	46	45-46		5				–	-/26	–
3	63	M	O	a	1	10%	46	34-48		7				–	-/38	–
4	64	M	B	a	1	10%	43	40-90		19	1	5		+	-/30	–
5	65	M	A	a(m)	1	–	57	35-71		8	8			–	1/29	–
6	66	M	B	a	1	10%	43	30-46		10				–	-/12	–
7	72	F	A	a	1	70%	30	19-39	2	3				–	1/54	–
8	70	M	A	a	1	–	46	40-47		20				–	2/9	–
9	58	F	A	a(m)	1	30%	43	40-45		16				–	2/16	+ (T1G3)
10	57	M	A	a(m)	1	30%	43	38-78		19	3			+	3/53	+ (T1G1)
11	41	M	A	a(m)	1	40%	45	45-46		5				+	4/24	–
12	59	M	B	a(m)	1	–	43	40-44		5				–	-/30	–
13	63	M	A	a	1	–	43	41-47		16				–	-/24	–
14	73	M	O	a	1	20%	48	46-48		8				–	-/12	–
15	55	M	A	a	1	–	44	43-46		7				–	1/30	–
16	17	M	A	a	1	–	46	45-46		5				–	1/48	–
17	77	M	O	a	1	10%	46	42-47		11				–	-/36	–
18	56	M	B	a	1	–	42	35-45		10				+	chemotherapy	
19	60	F	O	a	1	30%	46	32-49	10	74				+	-/42	–
20	58	M	O	a	1	40%	46	34-46		10				–	-/52	–
21	91	M	A	a(m)	1	10%	46	38-46		46				–	-/12	–
22	65	M	O	a	1	–	46	46		4				–	-/60	–
23	56	M	AB	a	1	20%	44	36-46		13				–	3/48	–
24	71	M	A	a	1	5%	46	35-48		5				+	-/66	–
25	61	M	O	a(m)	1	10%	40	30-46		5				–	2/66	–
26	63	M	O	a	1	30%	43	42-44		5				–	1/44	–
27	60	M	A	a	1	10%	46	44-46		10				–	-/12	–
28	76	M	A	a	1	90%	45	40-45		9				–	chemotherapy	
29	48	M	A	a	1	30%	45	37-46		15				–	-/18	–
30	65	M	O	a	2	–	42	19-45	11	16				+	4/24	+ (T1G2)

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Patient				Tumor			Chromosomal								Follow up	
Number	Age	Sex	Blgr[1]	T	G	B.G.I. Pos.[2]	Mode	Range	Ploidy[3] n	2n	3n	4n	5n	Marker	Rec./ Months[4]	Progression[5]
31	73	M	O	a	2	–	70	60-89			7			+	1/16	+ (T2G3)
32	82	M	A	a	2	5%	67	50-90		2	7	4		+	not fit for treatment	
33	61	M	O	a	2	–	64	50-75			20			+	1/54	–
34	72	F	O	a	2	–	45	38-57		11				+	-/20	–
35	66	M	A	a	2	10%	40	23-44	4	31				+	1/26	–
36	78	M	O	a	2	30%	45	39-87		31	1	2		+	1/25	–
37	44	M	O	a	2	–	46	36-49		27				+	chemotherapy	
38	83	M	O	a	2	–	78	40-83		13	13	1		+	chemotherapy	
39	55	M	O	1	1	5%	44	37-72		47	1			+	1/20	–
40	78	F	O	1(m)	2	10%	46	46-92		38	6	4		–	1/ 5	+(T2(m)G2)
41	66	M	O	1(m)	2	5%	65	35-72		4	7			+	1/ 4	+ (T2(m)G3)
42	77	M	O	1	2	20%	53	28-73	2	9	5			+	1/60	–
43	45	M	A	1	2	–	70	46-120		14	53	2		+	death unrelated to cancer	
44	59	M	A	1	2	–	42	35-72		38	2			+	-/40	–
45	72	M	B	1	2	–	72	62-77			25			+	chemotherapy	
46	53	M	A	1	2	–	43	35-57		13				+	radiotherapy	
47	54	M	O	1	2	20%	44	19-69	11	38	6			+	death unrelated to cancer	
48	62	F	O	1	2	5%	87	44-89		2	1	16		+	chemotherpay	
49	86	F	O	1	2	–	80	75-86			16	4		+	chemotherapy	
50	70	F	A	1	2	–	44	43-70		18	6			+	chemotherpay	
51	74	M	B	1	2	–	47	44-86		21		1		–	chemotherapy	
52	81	M	O	1	2	30%	84	75-88				18		–	chemotherapy	
53	69	F	O	1(m)	2	–	45	23-80	4	7	4			–	1/ 4	+ (T3G3)
54	69	M	O	1	2	–	46	30-87		32	1	2		+	1/ 8	+ (T2G3M1)
55	73	F	O	1	3	–	73	70-89			3	3		+	radiotherapy	
56	68	M	O	1	3	–	90	86-100			5			–	radiotherapy	
57	45	M	A	1	3	–	70	65-90			5	2		–	-/ 9	–
58	60	M	O	1	3	–	88	43-100	1	1	13		–	1/ 3	–	–
59	65	M	A	1	3	–	146	43-147	1	1	1	4	+	1/ 5	–	–
60	73	M	A	2	2	–	46	43-75		27	6			–	-/24	–
61	84	M	O	2	2	–	72	30-89	1	2	15	2		+	radiotherapy	

Histopathologic and cytogenetic data of individual bladder tumor patients

Patient				Tumor				Chromosomal							Follow up	
Number	Age	Sex	Blgr[1]	T	G	B.G.I. Pos.[2]	Mode	Range	Ploidy[3] n	2n	3n	4n	5n	Marker	Rec./ Months[4]	Progression[5]
62	82	M	O	2	3	–	58	19-93	3	3	3			+	radiotherapy	+ cystectomy
63	71	F	A	2	3	–	44	42-70		5	1			–	radiotherapy	+ cystectomy
64	56	M	O	2	3	–	46/90	46-90		3	1	4		–	radiotherapy	+ cystectomy
65	70	F	O	2(m)	3	–	69	36-90		17	2	10		+	radiotherapy	
66	76	M	A	2	3	–	45/90	30-90		20		24		+	death unrelated to cancer	
67	66	M	A	3	2	–	69	46-100		1	3	1		-	radiotherapy	
68	72	M	O	3(m)	2	–	46	40-69		4	2			+	cystectomy	
69	78	M	O	3	2	–	73	65-80			13			+	radiotherapy	
70	73	M	B	3	2	–	46	42-62		9	1			–	radiotherapy	
71	69	M	O	3	2	–	56	46-90		6		2		–	cystectomy	
72	75	M	A	3	3	–	92	90-95				5		+	radiotherapy	
73	73	M	A	3	3	–	45	42-90		3	2			–	cystectomy	
74	56	M	O	3(m)	3	–	75	64-90			5	5		+	radiotherapy	
75	74	F	A	3	3	–	70	50-90			4	1		+	radiotherapy	
76	64	M	O	3	3	–	90	19-69	1	4	4	13		–	death of cancer	
77	60	M	A	4(m)	2	–	43	40-140		19	4	1	5	–	radiotherapy	
78	44	F	B	4	3	–	56	46-120		8	3	1		–	radiotherpay	

[1] Blgr : bloodgroup

[2] B.G.I. pos. : estimated percentage of tumor area in slide in which by microscopic examination tumor cell membrane staining can be seen

[3] Ploidy : $n = 23 \pm 11$; $2n = 46 \pm 11$; $3n = 69 \pm 11$ etc.

[4] Rec./Months : number of recurrences per total number of months follow up

[5] Progression : progression in tumor stage

Chapter VII

General discussion

Histopathological grading

The most important parameters for prognosis in superficial transitional cell carcinomas of the urinary bladder are tumor stage and grade.[1-5] Grading of these tumors lacks an objective basis and therefore appears to be inconsistent.[6] The primary aim of this study was the search for parameters giving a sharper histopathological distinction between low and intermediate grade carcinomas. To find additional prognostic parameters we examined tumors cytogenetically and with respect to blood group antigen deletion.

The cytological abnormalities considered as evidence of anaplasia are summarized in the WHO-booklet on grading in bladder tumors.[7] Among pathologists increased cellularity only is generally considered as the least degree of anaplasia. This means that tumors showing only thickened epithelium without appreciable cellular deviations are to be defined as grade 1.[8,9] We found no difference between tumors showing only increased cellularity and those showing in addition more evidence of cellular atypia as some irregularity in size of cells and nuclei and slight polymorphism neither with respect to tumor stage nor to clinical course. Tumors with these abnormalities in otherwise cystoscopically normal bladders, were mostly non-invasive and not accompanied by signs of severe dysplasia elsewhere in the bladder. The clinical course of these tumors appeared to be rather innocent. The recurrence probability within 3 years was 43 per cent, during the first year after initial tumor resection in non-invasive tumors only 5 per cent. Tumor progression was present in only 5 per cent of cases during our limited follow up of two years.

In our view, the group of low grade or grade 1 transitional cell carcinomas consists of urothelial cell tumors showing increased cellularity with or without mild cellular atypia. Cystoscopic control of these tumors can be postponed till at least half a year after initial diagnosis, provided that only a single tumor was found. Prophylactic intravesical chemotherapy or immunotherapy should be reserved for those patients showing multifocal disease or recurrences.

In our opinion, as grade 2 TCC ought to be considered those neoplasms showing microscopically thickened epithelium with distinct cellular irregularity, clear variations in nuclear and cellular size, quite noticeable hyperchromasia, and an evident tendency to loose the normal polarity of epithelial cells. These tumors can be non-invasive but in majority they appeared to be submucosally or deeply invasive. Sometimes they were accompanied by severe dysplasia in non-tumor bearing areas in the bladder. Clinically, at least 30 per cent of these tumors was at risk for a recurrence during the first year after initial treatment, which figure increased to more than 80 per cent within 3 years. Tumor progression was seen in one out of every three superficial tumors graded as intermediate according to our criteria, often rather early after initial diagnosis.

The proposed grading criteria offer a consistent distinction between low and intermediate TCC with clinical relevance. The grading criteria applied in our study appeared also liable to some intra- and interindividual inconsistency. Quantitative morphometry has been

applied to try to characterize the different grades of human bladder carcinoma more objectively.[10-13]

Chromosomal numbers

Cytogenetic analysis of bladder tumors was done to investigate the prognostic significance of chromosomal abnormalities and their correlation with our histopathological grading criteria in TCC. Initially, with conventional staining methods recognizable chromosomes were found in only 45 per cent of the tumors examined (this thesis chapter II). Due to technical improvements, at present recognizable metaphases are found in 93 per cent of the tumor biopsies, irrespective of tumor stage.[14] Identification of chromosome abnormalities with chromosome banding methods remains a problem. This regards especially the non-infiltrating bladder tumors where the yield of analyzable metaphases per tumor biopsy is usually lower than in invasive ones. Chromosomal analysis of cultured cells may reveal more karyotypic abnormalities.[15-17] However, such a technique involves the risk of selection of particular cell types during culture.

The majority of low grade and non-invasive tumors have been reported to have near-diploid modal numbers and normal DNA values, while high grade and deeply invasive tumors showed frequently hyperdiploid modal numbers and abnormal DNA values.[18-25] The large group of so-called intermediate grade superficial tumors appeared to be heterogeneous. It is in this group that objective parameters or additional tumor markers are urgently needed. Till now the discriminating power of DNA cytometry is deficient in these tumors.[26] Small numerical or structural aberrations which do not change the mass of DNA rigorously cannot be recognized by flowcytometric DNA analysis. By investigating the relation between the range in chromosomal numbers and tumor stage and grade, we saw remarkable correlations. All invasive tumors, even if only superficial (pT1), contained at least a few hyperdiploid cells, while the modal numbers often were (near) normal. Therefore, the range in chromosomal counts appeared to be a better reflection of invasion than the modal number.
Regarding tumor grade, we found normal chromosome numbers in grade 1 tumors, in majority hyperdiploid modal numbers in grade 3 tumors. The group of intermediate grade tumors was heterogeneous with respect to modal numbers. However, these tumors nearly always showed a hyperdiploid chromosomal range. The correlation with chromosomal range gives a biological meaning to our grading system, namely a distinction between those superficial tumors which appear less likely to impose a risk to a patient with bladder cancer, and those which are biologically more aggressive. Like most solid tumors, individual bladder tumors often show heterogeneities, with respect to histology [27-29] as well as with respect to ploidy [30,31] and other tumor markers.[24,32,33] By single-cell cytophotometric analysis of urothelial bladder cancer it was demonstrated that the superficial portion of Ta or T1 tumors contained a relatively higher percentage of diploid tumor cells than did the deeper, basaloid regions.[31] Apparently this heterogeneity of tumor cells is better reflected in the range of chromosomal numbers than in a sharp modal number. This cytogenetic heterogeneity might permit selection and increase of aberrant cells that are responsible for tumor progression and metastasis.[34] The conclusion may be that in superficial bladder cancer the discriminating power of chromosomal range, with regard to tumor progression, is better than that of modal number and DNA-value.

There may have been a bias for tumors of larger size in the cytogenetically analyzed cancers. We needed tumor material for routine histopathological, immunohistochemical, cytogenetical and flowcytometrical studies. Especially during the first years of the cytogenetic analysis, fairly large amounts of tumor material were needed for cytogenetic examinations. Thus, in the beginning very small tumors were excluded from chromosomal analysis.[35] According to some authors, large size of a bladder tumor at the initial assessment is associated with a poorer prognosis.[3,5,36] This could explain why the clinical course of patients examined cytogenetically seems worse than we reported in the study on morphological grading criteria.

Marker chromosomes

Cytogenetic studies can show two kinds of chromosomal abnormalities in cancer: numerical and structural. Marker chromosomes belong to the latter group. They are structural abnormalities which cannot be identified unless specific banding techniques are applied. Especially in poorly differentiated and invasive bladder cancers marker chromosomes are frequently reported.[22,37,38] Markers were regarded as a very bad prognostic factor.[22,39-42] It was said that, in general, tumors without markers could be expected to behave in a noninvasive fashion, while tumors with markers could be expected to produce numerous recurrences and to become invasive.[42] The combination of markers, a hyperdiploid mode and submucosal invasion should involve a lethal prognosis and mandate radical bladder resection.[40,41]

In our study more abnormal chromosomes were found in higher graded tumors or those with a hyperdiploid chromosomal range. The finding of unidentified abnormal chromosomes had no prognostic value additional to tumor grade or chromosomal numbers. With respect to tumor progression no significant difference between superficial tumors with or without marker chromosomes was observed. Thus, in our opinion the finding of marker chromosomes in non-banded preparations, does not have the predictive value generally[22,37-42] assigned to this parameter in superficial bladder cancer. The low frequency of abnormal chromosomes in superficial cancer as mentioned by earlier authors probably is due to technical shortcomings of chromosome preparations.[14]

For identification of the different changes in chromosomal structure, banding techniques are necessary. Using these techniques, rather specific cytogenetic findings have been detected for a variety of solid and hemopoietic neoplasms.[43-47] We applied G-banding succesfully in 13 bladder tumors at first diagnosis. Seven of these were superficial tumors of low and intermediate grade. Chromosome # 9 was involved in four of these superficial cases. A missing chromosome # 9 in diploid bladder tumors has been reported by other authors.[47-52] These data suggest that loss of chromosome # 9 may be a primary karyotypic change in bladder cancer. Primary chromosomal changes are possibly related to tumor cell etiology.[53,54] In the more advanced, infiltrating, hyperdiploid tumors, we found many changed chromosomes. The karyotypes of these tumors were very different. Probably the increasing variation in chromosome number is secondary and occurs during the progression of the neoplasia. The correlation with more advanced tumors suggests that such secondary chromosomal changes contribute to the capacity of the tumor to invade, metastasize and kill the host. It has been suggested that these secondary chromosomal changes, often resulting in extra gene dosage, confer proliferative advantage to some clones of tumor cells.[16] So far cytogenetic characterisation of aberrations that may be indicators for infiltration and dissemination is an objective that remains to be fulfilled.

The application of advanced banding techniques on non-cultured cells in larger series of bladder tumors with a longer follow up is needed to see what exactly is the prognostic significance of structural chromosomal abnormalities in bladder cancer.

ABH antigen deletion

At the start of this study on the ABH antigen status in bladder tumors, it was generally assumed that determination of ABH antigen expression in urinary bladder carcinoma had predictive value.[55-61] With respect to tumor grade, some authors mentioned a significant correlation between histopathological grade and blood group antigen status.[55,57,58,60] Others reported that presence or absence of blood group antigens in bladder tumors was not merely a reflection of histological grade.[62-66] A number of techniques was available to demonstrate these antigens, for instance the specific red cell adherence test [55,67], the immunofluorescence technique [68-70] and the immunoperoxidase method.[61,69,71] The conflicting results might, at least partly, be due to differences in methodology and interpretation. Also the lack of uniformity in tumor grading could be of relevance.

The availability of monoclonal antibodies might solve some of the problems of other tests, especially because of their absolute purity and well defined specificity. In the present study the ABH antigen status as found by the indirect immunoperoxidase method using monoclonal antibodies, was compared with tumor grade and stage. The demonstration of antigens by immunoperoxidase procedures depends on the development of contrast between the staining of the antigen and its surroundings. In cell surface studies the optimum concentration of antibodies is determined by the dilution beyond which further twofold dilution produces no additional fall in the percentage of positive cells.[72] In our laboratory, for normal urothelium this was a dilution of the appropriate monoclonal antibody of 1:100. Using this concentration, we retained a high degree of sensitivity for normal urothelium and vascular endothelium, while background staining was very low, never exceeding 5 per cent.
With this method, the pattern and intensity of blood group antigen-antibody staining in superficial tumors appeared very variable. All deeper invasive and high grade tumors were however completely ABH negative. A significant difference in BGI-expression was seen between low grade tumors versus intermediate and high grade tumors. Therefore the mode of expression of these antigens clearly reflects signs of malignant dedifferentiation.

Significantly more superficial grade 2 tumors were negative for blood group antigens than grade 1 tumors. Thus it might be possible that the mode of BGI expression in superficial tumors has additional prognostic value. However, no consistent correlation between ABH status and clinical course was found, neither with respect to tumor recurrences, nor to progression. This confirms recent observations made by other authors.[36,73]

From our study it is clear that there is a direct correlation between higher histological grade and loss of bloodgroup antigens. In superficial tumors the mode of BGI expression is variable and difficult to express on a quantitative way. As long as there is no general consensus about standardisation of the technique and quantitative interpretation of test results, the ABH antigen status in superficial TCC of the bladder cannot be used prognostically or therapeutically in an individual patient.

Arranging the test results on an arbitary scale according to the percentage of cells considered positive for antigen staining, remains a superficial interpretation of the

heterogeneity of bloodgroup antigen staining in bladder tumors. Recently, a method was described to record the staining intensity on a score in accordance with the dilution of antibody needed to give a clearly positive coloration for the antibody-antigen reaction.[74] However prediction of an unfavourable course with this assay appeared not possible. It has been suggested that reagents recognizing bloodgroup precursor substances, common to all individuals irrespective of the ABO and saliva secretor types, may increase the prognostic accuracy of bloodgroup antigen determination in bladder cancer.[74] This has to be investigated.

Determinants of the A, B and H bloodgroup antigens are carbohydrate structures carried on both glycoproteins and glycolipids.[75,76] Incomplete synthesis or partial degradation of olygosaccharide chains can thus result in both deletion of normal blood group antigens and expression of abnormal precursor substances. Biochemical analysis of these antigenmolecules permit quantitative analysis and precise delineation of molecule structures.[77] A significant limitation of biochemical studies is that they require samples of fresh tissue, in which the amount of viable tumor tissue may be highly variable. This makes biochemical analysis of BGI expression in bladder carcinomas in clinical practice difficult to realize in a reproducible way.

Multiparameter flowcytometric analysis of blood group antigens in tumor cells may be a promising technique.[81] Urothelial cells in diploid tumors might be recognized by labeling the cell suspensions with antibodies to cytokeratins.[26,83]

Alterations in the ABH antigens may occur relatively early in tumor progression. ABH deletion has been described in premalignant epithelium in the bladder.[78-82] Blood group antigen deletion might thus be indicative of the presence of a malignant diathesis in apparently normal urothelium prior to the morphologic expression of the neoplastic lesion. In a recent study, ABH antigenicity of mucosal biopsies proved to be a better predictor of recurrent disease that other prognostic indices.[73]

Immunohistochemical studies have also explored the relation between the expression of other blood group antigens and the biological behavior of transitional cell carcinoma.[24,76,84-86] The prognostic significance of presence or absence of these blood group related antigens in superficial TCC still has to be defined.

Probably testing for blood group related antigens in bladder cancer is more appropriate for making therapeutic decisions as soon as the biochemical, biophysical and cellular factors which influence the expression of these antigens are better understood. In conjunction with other tumor markers testing for blood group related antigens might then be of help to identify specific risk factors regarding the future behavior of a bladder tumor in an individual patient.

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Chapter VIII

Summary and conclusions

Superficial bladder cancer has a natural course uncertainly to predict. Most important prognostic factors are tumor stage and grade. Aim of this study was the search for factors contributing to a sharper histopathological distinction between low and intermediate grade carcinomas and to find additional prognostic parameters.
For this purpose, chromosomal changes, microscopic histopathology and ABH antigen-deletion were studied. Initially, recognizable chromosomes were found in only 45 per cent of the tumors examined, at present due to technical improvements in 93 percent. Non-invasive tumors nearly always were neardiploid. In all invasive cancers at least some cells with many extra chromosomes were found. The range in chromosomal counts appeared to be a better reflection of invasion than the modal number.
In tumors with a (near)-normal chromosomal mode, those with chromosome counts up to the hyperdiploid range tended to behave worse than tumors with only near normal numbers: they recurred earlier and showed significantly more often tumor progression. Thus, with respect to the clinical course, the discriminating power of the chromosomal range was better than that of the modal number. The finding of chromosomal markers, unidentified structurally abnormal chromosomes, had no prognostic value additional to tumor stage, grade or chromosomal number.
The data obtained with chormosome analysis using G- and C- banding suggest that the loss of chromosome # 9, and possibly also of the long arm of chromosome #10, is a primary event in the karyotypic evolution of TCC. The increase in number of chromosomes is probably secondary and occurs during the progression of the neoplasia.

In a prospective study on grading of superficial bladder cancers, a histopathological distinction was made within the group of, initially called, intermediate grade tumors. According to this study tumors with some cellular variation (grade "2a") and those showing only increased cellularity both are to be regarded as grade 1 TCC. They behave significantly different from those cancers showing clear cytologic deviation and a tendency to loose the normal polarity (grade "2b"). Grade 1 tumors according to our criteria generally were noninvasive. Their chance of recurrence during the first 3 years after initial endoresection was less than 50 percent. If recurrence happened, it usually took place late. Tumor progression was seldom seen. Grade 1 tumors had near diploid chromosomal numbers and were mostly positive for ABH blood group antigens.

Tumors which according to this study have to be regarded as grade 2 TCC were often submucosally or deeply invasive, had a recurrence probability of more than 80 percent within 3 years after initial endoresection, recurred often early, and showed progression in at least 30 percent. Tumors thus graded had chromosomal counts up to the hyperdiploid range, although half of them showed near normal modal numbers. These cancers were mostly negative for ABH blood group antigens.

Grade 3 TCC applies to tumors with the most severe degree of cellular anaplasia. They were in majority deeply invasive, had hyperdiploid modal chromosome numbers, and were negative for ABH blood group antigens.

In this study the ABH antigen status, as found by the indirect immunoperoxidase method, using monoclonal antibodies, was compared with tumor stage and grade and clinical course.
The pattern and intensity of blood group antigen - antibody staining in superficial tumors appeared very variable and quantitatively difficult to express. A significant difference in BGI-expression was seen between low grade - low stage versus intermediate or high grade and invasive tumors. With the method used in this study, prediction of the clinical course in superficial TCC according to BGI-deletion appeared not possible.
A general consensus about standardization of the technique and quantitative interpretation of test results with respect to blood group related antigens in tumors is needed to use this assay prognostically or therapeutically in an individual patient.

Samenvatting en conclusies

Het natuurlijk verloop van oppervlakkige blaastumoren is moeilijk voorspelbaar. De belangrijkste parameters met betrekking tot de prognose zijn stadium en graad van de tumor. Doel van deze studie was het zoeken naar een consistent histopatologisch onderscheid tussen graad 1 en graad 2 tumoren, alsmede naar andere prognostische parameters. Hiertoe werd chromosomaal en microscopisch histopathologisch onderzoek gedaan en de ABH bloedgroep antigenen expressie werd immunohistochemisch onderzocht.
Aanvankelijk werden slechts in 45 procent van de tumoren beoordeelbare metafasen verkregen, welk percentage thans dank zij technische verbeteringen in de methode van onderzoek is gestegen tot 93. Niet infiltrerende tumoren bleken bijna steeds een nagenoeg normaal aantal chromosomen te hebben. In infiltrerende tumoren werden vrijwel steeds cellen met extra chromosomen aangetroffen. De spreiding van het aantal chromosomen bleek een betere weerspiegeling van invasieve groei te zijn dan het modale aantal chromosomen.
Bij tumoren met een vrijwel normaal modaal aantal chromosomen had de aanwezigheid van cellen met een hyperdiploid aantal in het algemeen een slechtere prognostische betekenis. Dergelijke tumoren recidiveerden eerder en toonden significant vaker progressie. De spreiding van het aantal chromosomen heeft dus een grotere prognostische waarde dan het modale aantal. In tegenstelling tot wat hieromtrent door eerdere auteurs werd vermeld voegde het vinden van niet geïdentificeerde, structureel afwijkende chromosomen niets toe aan de verwachting van de prognose op basis van stadium, graad en chromosomenaantal van de tumor.
Chromosomaal onderzoek met behulp van G- en C-bandering suggereerde dat het verlies van chromosoom # 9 en mogelijk ook van de lange arm van chromosoom # 10, een primaire afwijking is in de oncogenese van de blaas. De toename van het aantal chromosomen is mogelijk een secundaire gebeurtenis tijdens de verdere evolutie van een blaastumor.

In een prospectief onderzoek betreffende gradering van het blaascarcinoom werd, naar microscopisch histopathologische criteria, een onderscheid gemaakt binnen de groep van de tot dan matig gedifferentieerd genoemde tumoren. Tumoren met geringe cellulaire en nucleaire afwijkingen (graad "2a") toonden klinisch een zelfde gedrag als tumoren welke alleen celtoename lieten zien. Dit gedrag was significant anders bij tumoren met duidelijke cytologische afwijkingen bij welke de normale epitheliale opbouw enigermate was verstoord (graad "2b"). Met betrekking tot de prognostische betekenis dienen tumoren welke alleen een toename van het aantal cellen vertonen en die welke daarenboven geringe cytologische atypie hebben, als één groep te worden beschouwd, namelijk de graad 1 urotheelcel tumoren. Deze maligne nieuwvormingen waren in het algemeen niet infiltrerend. De kans op een recidief binnen 3 jaar na verwijdering was kleiner dan 50 procent. Recidieven van graad 1 tumoren werden in het algemeen laat gezien, eerst een jaar of meer na endoresectie. Zij hadden een nagenoeg normaal aantal chromosomen en in meerderheid hun ABH bloedgroepantigenen behouden.
Als graad 2 urotheelcel-carcinomen dienen te worden beschouwd die tumoren welke naast

celtoename een duidelijke variatie in vorm en afmeting van cel en kern tonen en een neiging hebben de normale epitheel-opbouw te verliezen.
Zij groeiden vaak submuceus of dieper infiltrerend, hadden een recidiefkans van meer dan 80 procent binnen drie jaar na eerste behandeling, recidiveerden vaak al vroeg, en toonden in meer dan 30 procent van de gevallen progressie. Tumoren met deze mate van dedifferentiatie hadden in de helft van de gevallen een nagenoeg normaal modaal aantal chromosomen. Bij deze gevallen werden steeds cellen gevonden met een hyperdiploidie. Deze neoplasmata waren meestal negatief voor ABH bloedgroep-antigenen.
Tot de graad 3 urotheelcel-carcinomen behoren tumoren welke de ernstigste mate van cellulaire atypie tonen. Zij waren meestal diep infiltrerend, hadden hyperdiploide aantallen chromosomen, en waren altijd negatief voor ABH bloedgroep-antigenen.

In deze studie werd de wijze van expressie van ABH bloedgroepantigenen bepaald met behulp van de indirecte immunoperoxidase methode met gebruikmaking van monoclonale antilichamen. De bloedgroep antigenen-expressie werd gerelateerd aan stadium, graad en klinisch verloop van de tumor. Het patroon en de intensiteit van kleuring van bloedgroep-antigenen in oppervlakkige blaastumoren bleek zeer variabel en moeilijk in een exacte maat uit te drukken. Tussen niet-infiltrerende graad 1 tumoren enerzijds en graad 2 en 3 en invasief groeiende tumoren anderzijds, werd een significant verschil in BGI-expressie gezien. Voorspellen van het klinisch verloop van een oppervlakkige blaastumor bleek met de in deze studie gebruikte methode echter niet mogelijk. Standaardisatie van de methode en haar interpretatie is vereist alvorens deze techniek bruikbaar is met betrekking tot prognose en therapieplanning voor een individuele patient.

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Curriculum vitae

De schrijver werd in 1937 te Heerlen geboren. Na het eindexamen Gymnasium B aan het Henric van Veldeke College te Maastricht in 1956, studeerde hij geneeskunde aan de Katholieke Universiteit te Nijmegen. In 1962 werd het doctoraalexamen geneeskunde afgelegd, in 1964 volgde de bevordering tot arts. In 1964-1965 nam hij waar in meerdere huisartsenpraktijken, waarna hij van 1965-1969 assistent was op de afdeling Algemene Chirurgie van het St. Radboud Ziekenhuis te Nijmegen (opleiders: prof. dr E.J. Moeys; dr D. de Moulin; prof. dr W.J.H. Schmidt). Hij werd opgeleid tot uroloog aan het Groot Zieken Gasthuis te 's-Hertogenbosch (opleider: prof. dr W.A. Moonen), van 1969-1971, waarvan een viertal maanden aan het Institute of Urology te Londen. Inschrijving in het specialisten-register als uroloog geschiedde op 1 juli 1971. Sindsdien is hij als uroloog verbonden aan het St. Jozef Ziekenhuis, later St. Maartens Gasthuis, te Venlo, waar hij van 1974-1977 en van 1979-1981 vice-voorzitter was van het stafbestuur. Hij is lid van de Société Internationale d'Urologie, Corresponding Member of the American Urological Association en, sinds 1985, bestuurslid van de Nederlandse Vereniging voor Urologie.

Stellingen

1. Bij oppervlakkige blaastumoren levert het aantonen van hyperdiploide cellen een juistere prognostische parameter dan het modale aantal chromosomen of de D.N.A.-waarde.

2. Markerchromosomen zijn vooralsnog niet bruikbaar als additionele tumormarkers.

3. Indien papillaire, solitaire, niet-infiltrerende urotheelceltumoren bij eerste diagnose niet als zodanig herkend worden, dient het nemen van multiple biopten uit ogenschijnlijk normaal blaasslijmvlies te worden overwogen.

4. Bij het voornemen tot prophylactische behandeling van papillaire, solitaire, niet-infiltrerende, goed gedifferentieerde blaastumoren met intravesicale chemotherapeutica of immunostimulerende agentia dient het betrekkelijk goedaardig verloop van de meeste van deze tumoren mede in overweging genomen te worden.

5. De bruikbaarheid van het onderzoek naar het verlies van bloedgroep-antigenen bij het blaascarcinoom wordt ernstig beperkt door het ontbreken van overeenstemming omtrent de kwantitatieve interpretatie van betreffende testresultaten.

6. Onder de verschillende methoden ter afleiding van de urinewegen bij een radicale cystectomie dient de uretero-ileo-cutaneostomie vooralsnog de voorkeur te genieten.

7. Circumcisie bij neonaten kan een belangrijke rol spelen ter preventie van nierinsufficiëntie op latere leeftijd.

8. De adequate behandeling van ongecompliceerde urineweginfecties bij vrouwen vóór de menopauze middels kortdurende antibiotische therapie vormt meer een diagnostisch dan een therapeutisch probleem.

9. Bij regionalisatie van gezondheidszorg dient een provinciegrens geen belemmerende factor te zijn.

10. Het vastleggen van een studie in een proefschrift mag geen stagnerende factor vormen voor verder onderzoek.

11. Het verdient aanbeveling om "Götterdämmerung" van Wagner in disco-versie op te nemen.

4 september 1987, R.P.E. Pauwels.